

The #MeToo provocateur
shaking up STEM p. 682

Learning like a
human p. 692

Transcribing through
the nucleosome p. 744

Science

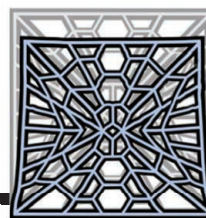
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GORDON RESEARCH CONFERENCES

Topics include ecological
genomics, bioelectronics, and
bacterial pathogens p. 758



NEWS

IN BRIEF

672 News at a glance

IN DEPTH

675 DOE TO LIMIT FOREIGN RESEARCH COLLABORATIONS

Recent memos describe crackdown on grantees and on lab scientists with foreign ties *By J. Mervis*

676 CONTROVERSIAL FLU STUDIES CAN RESUME, U.S. PANEL SAYS

Critics complain of lack of transparency in decision that allows efforts to create potentially risky virus strains *By J. Kaiser*

677 MEASLES EPIDEMIC IN UKRAINE DROVE TROUBLING EUROPEAN YEAR

War and distrust of vaccines seeded ongoing outbreak *By M. Wadman*

678 HOW RABBITS ESCAPED A DEADLY VIRUS—AT LEAST FOR NOW

Same genetic changes found in rabbits on two continents *By E. Pennisi*

► RESEARCH ARTICLE BY J. M. ALVES ET AL.
10.1126/science.aau7285

679 IN INDIA, HINDU PRIDE BOOSTS PSEUDOSCIENCE

Claims of great technological achievements in ancient times trigger ridicule and concern *By S. Kumar*

680 AIDS PUSH GETS MIXED REVIEWS

Trump's initiative to "end AIDS" promises money, focus *By J. Cohen*

681 METRIC PREFIXES SOUGHT FOR EXTREME NUMBERS

"Ronna" and "quecca" would help computer scientists keep pace with big data *By D. Adam*

FEATURES

682 THE TWITTER WARRIOR

#MeToo provocateur BethAnn McLaughlin battles on behalf of women in STEM—but her own job is in peril *By M. Wadman*



682

INSIGHTS

POLICY FORUM

686 LONG DELAYS IN BANNING TRADE IN THREATENED SPECIES

Scientific knowledge should be applied with more urgency
By E. G. Frank and D. S. Wilcove

PERSPECTIVES

689 REVEALING A MICROBIAL CARCINOGEN

An *E. coli*-derived colibactin-DNA adduct is detected in intestinal tissues
By R. M. Bleich and J. C. Arthur
► RESEARCH ARTICLE P. 709

690 PATHOLOGY-LINKED PROTEASE CAUGHT IN ACTION

Structural snapshots of γ -secretase yield insight for drug development
By S. F. Lichtenthaler and G. Güner
► RESEARCH ARTICLE P. 708

692 USING NEUROSCIENCE TO DEVELOP ARTIFICIAL INTELLIGENCE

Combining deep learning with brain-like innate structures may guide network models toward human-like learning *By S. Ullman*

694 HYPERBOLIC 3D ARCHITECTURES WITH 2D CERAMICS

A hexagonal boron nitride aerogel has high resistance to thermal and mechanical shock
By M. Chhowalla and D. Jariwala
► REPORT P. 723

695 MOVING THROUGH THE CROWD

Atoms or molecules can diffuse rapidly on surfaces covered by other adsorbants
By O. M. Magnussen
► REPORT P. 715

696 EARTH'S RUGGED LOWER MANTLE

Seismic data reveal kilometer-scale topography of the lower-mantle boundary *By C. Houser*
► REPORT P. 736

698 ROY GLAUBER (1925–2018)

Father of quantum optics
By *M. O. Scully and S. Prasad*

BOOKS ET AL.**699 CONSERVATION AND CONFLICT**

Afghanistan's rich natural heritage shines in an insider's account of the foundations of its first national parks
By *R. Cooney and K. Karimov*

700 OVERLOOKED NO LONGER

A new play honors the enslaved black women who helped improve our understanding of reproductive health
By *D. Cooper Owens*

LETTERS**701 SAVING CHINA'S ONAGER**

By *Y. Li et al.*

701 HEMP HEMP HOORAY FOR CANNABIS RESEARCH

By *C. Schluttenhofer and L. Yuan*

702 ARGENTINA'S SUBPAR INVESTMENT IN SCIENCE

By *H. A. Carignano and J. P. Jaworski*

**RESEARCH****IN BRIEF**

703 From *Science* and other journals

REVIEW**706 MAGNETISM**

Two-dimensional magnetic crystals and emergent heterostructure devices
By *C. Gong and X. Zhang*

REVIEW SUMMARY; FOR FULL TEXT:

dx.doi.org/10.1126/science.aav4450

RESEARCH ARTICLES**707 NEURODEGENERATION**

Heterochromatin anomalies and double-stranded RNA accumulation underlie *C9orf72* poly(PR) toxicity
By *Y.-J. Zhang et al.*

RESEARCH ARTICLE SUMMARY; FOR FULL TEXT:

dx.doi.org/10.1126/science.aav2606

708 STRUCTURAL BIOLOGY

Recognition of the amyloid precursor protein by human γ -secretase
By *R. Zhou et al.*

RESEARCH ARTICLE SUMMARY; FOR FULL TEXT:

dx.doi.org/10.1126/science.aaw0930

► PERSPECTIVE P. 690

709 TOXINS

The human gut bacterial genotoxin colibactin alkylates DNA
By *M. R. Wilson et al.*

RESEARCH ARTICLE SUMMARY; FOR FULL TEXT:

dx.doi.org/10.1126/science.aar7785

► PERSPECTIVE P. 689

710 STRUCTURAL BIOLOGY

A human postcatalytic spliceosome structure reveals essential roles of metazoan factors for exon ligation
By *S. M. Fica et al.*

REPORTS**715 SURFACE SCIENCE**

Density fluctuations as door-opener for diffusion on crowded surfaces
By *A.-K. Henß et al.*

► PERSPECTIVE P. 695

719 DEVICE TECHNOLOGY

Printed subthreshold organic transistors operating at high gain and ultralow power
By *C. Jiang et al.*

723 CERAMICS

Double-negative-index ceramic aerogels for thermal superinsulation
By *X. Xu et al.*

► PERSPECTIVE P. 694

728 QUANTUM OPTICS

Photoelectrical imaging and coherent spin-state readout of single nitrogen-vacancy centers in diamond
By *P. Siyushev et al.*

731 NANOMATERIALS

Chemically reversible isomerization of inorganic clusters
By *C. B. Williamson et al.*

736 GEOPHYSICS

Inferring Earth's discontinuous chemical layering from the 660-kilometer boundary topography
By *W. Wu et al.*

► PERSPECTIVE P. 696

740 STRUCTURAL BIOLOGY

How a circularized tmRNA moves through the ribosome
By *C. D. Rae et al.*

744 STRUCTURAL BIOLOGY

Structural insight into nucleosome transcription by RNA polymerase II with elongation factors
By *H. Ehara et al.*

748 IMMUNOLOGY

BCR-dependent lineage plasticity in mature B cells
By *R. Graf et al.*

753 STRUCTURAL BIOLOGY

Structural insight into substrate and inhibitor discrimination by human P-glycoprotein
By *A. Alam et al.*

DEPARTMENTS**671 EDITORIAL**

Keep science on the horizon
By *Sir John Beddington*

786 WORKING LIFE

Waiting for reimbursement
By *Jessica Sagers*

ON THE COVER

In winter, snowshoe hares (*Lepus americanus*) molt from brown to white coats to maintain camouflage in snow-covered environments. This seasonal adaptation has evolved in several

animal species and can result in camouflage mismatch as snow duration decreases. The Gordon Research Conference on Ecological and Evolutionary Genomics will be held from 14 to 19 July 2019 in Manchester, New Hampshire. See page 758 for the Gordon Research Conference schedule and preliminary programs. *Photo: Colin Ruggiero*

Science Staff	670
Gordon Research Conferences	758
New Products	782
Science Careers	783

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Keep science on the horizon

Science is a global endeavor that affects well-being and stability on many fronts, so anything that damages the scientific enterprise in one country, particularly a powerhouse that is internationally networked, damages conditions everywhere. As the United Kingdom tumbles toward the 29 March Brexit deadline, the world should feel uneasy, at the very least. Paradoxically, science has never been high on the Brexit agenda likely because there is general agreement among politicians on all sides that good science relations are in everyone's best interest. Thus, the United Kingdom does not have a plan in place for preserving science. Everyone who cares about the future of science needs to continue to make the case for the smoothest transition on 29 March—whether there is a deal or not—so that U.K. science and global science do not become accidental victims.

U.K. science has always thrived on its internationalism. It has been open to the brightest people, inviting them to work alongside home-grown talent on innovative ideas. One in six academic staff in U.K. higher education institutions are from the European Union (EU). At the same time, the United Kingdom has exported talent around the world to learn and collaborate. Support for leaving the EU has remained spectacularly low in the U.K. science community. And yet, 6 weeks from now, science may suffer collateral damage due to the very complex politics of Brexit. A no-deal Brexit is the worst scenario. It would damage the United Kingdom's ability to collaborate and to access hugely valuable funding from EU research schemes. And it would send a message, intentional or not, and true or not, that the United Kingdom is no longer the open and welcoming country that it has always been.

A substantially better, if not optimum, scenario (which is arguably no Brexit at all) would see the United Kingdom effectively stay in Horizon 2020 during a transition phase, where researchers would continue to

move freely among countries for jobs and collaborations. Horizon 2020 is the largest EU Research and Innovation program ever established, providing nearly €80 billion of funding from 2014 to 2020. Leaving the EU without a deal may jeopardize the United Kingdom's access to the €1.3 billion that it has been receiving each year through this program.

What about Horizon Europe, which will raise EU science spending to approximately €100 billion over the years 2021 to 2027? For the longer term, the United Kingdom should ensure the closest possible association with Horizon Europe and a sensible approach to

immigration so that scientific progress continues smoothly. For overseas researchers who have jobs or grants that bring them to the United Kingdom, the United Kingdom must make it easy for them to bring their families and members of their research teams. The immigration system also must allow people to come and go on short-term visits for conferences or as part of collaborations. At the same time, the EU will have to ensure that Horizon Europe strengthens its international focus and remains open to genuinely constructive association status.

No one wants collateral damage to science, but that does not mean that it will not happen. Preserving Horizon 2020 and planning for Horizon Europe are actions that are distinctly

possible with the correct political will. Four months ago, 35 Nobel laureates and Fields medalists wrote to President Juncker of the European Commission and U.K. Prime Minister May, expressing concerns about the potential downside to European and world science if Brexit leads to a situation where the free interchange of science and science funding is threatened. National and global voices must assert more strongly that science is a high priority, and that no matter what the situation is come 29 March, the scientific enterprise must not be a victim of the political fallout.

—Sir John Beddington



Sir John Beddington
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at the Oxford
Martin School,
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*“...the scientific enterprise
must not be a victim
of the political fallout.”*

“One giant leap for womankind.”

Pop star Ariana Grande, in a new song, “NASA,” about matters celestial and romantic that earned shout-out tweets from the space agency and astronaut Buzz Aldrin.

IN BRIEF

Edited by Jeffrey Brinard

ASTRONOMY

Violence threatens Mexican scopes



Mexico's Large Millimeter Telescope reduced operations, which have included observations of black holes and nascent stars.

Two observatories in Mexico have reduced access and operations because of security threats, Mexico's National Institute of Astrophysics, Optics and Electronics (INAOE) in San Andrés Cholula announced last week. Both the Large Millimeter Telescope (LMT) and the High-Altitude Water Cherenkov Gamma-Ray Observatory (HAWC) are located on the Sierra Negra volcano in the state of Puebla. Organized crime groups that steal oil and gasoline from pipelines in the region have intensified highway robberies and carjackings as the government has cracked down on fuel theft. The LMT typically hosts scientists at night for observations and maintenance and engineering crews during the day. It was poised to start observations with a new 50-meter dish before what INAOE astrophysicist and LMT Director David Hughes calls “a severe security incident” caused him to dramatically reduce operations. He declined to describe the incident or say exactly what is being done to protect employees and collaborators. The HAWC, which studies cosmic rays, is operated remotely, which means it can continue to collect data. But planned equipment repairs have been canceled.

UC scores CRISPR patent win

BIOTECHNOLOGY | The University of California (UC) has received a critical patent for its invention of CRISPR, the enormously popular genome-editing tool. The U.S. Patent and Trademark Office notified UC last week that its patent had been “allowed” and should be issued soon, a step that may push the fierce legal war over this valuable intellectual property toward a treaty between sparring academic institutions. The protracted fight has pitted UC and its collaborators against a group led by the Broad Institute in Cambridge, Massachusetts. Broad received many CRISPR patents beginning in 2014. UC unsuccessfully challenged them, but its patent covers the fundamental invention of CRISPR as a research tool. Companies hoping to develop new medicines and crops may have to license from both groups, if they don't reach a settlement. Europe, meanwhile, has a separate patent system that so far has given UC an edge.

Holt to leave AAAS

SCIENTIFIC SOCIETIES | Rush Holt announced last week he will retire this fall as CEO of AAAS in Washington, D.C., and executive publisher of the *Science* family of journals. A physicist, Holt, 70, came to AAAS in 2015 after serving as a member of the U.S. House of Representatives for 16 years. While looking for a successor for Holt, AAAS is also recruiting an editor-in-chief for the *Science* journals; the current editor-in-chief, Jeremy Berg, announced in October 2018 that he will leave in June.

Small teams disrupt science

INNOVATION | Large teams have become common in many scientific fields in recent years. But a new study finds small teams are more likely to generate novel ideas, whereas heftier groups are more likely to build incrementally on established ideas. Researchers drew those conclusions, reported in a 13 February letter in *Nature*, by examining citation patterns for 65 million papers, patents, and software products appearing from 1954 to 2014. They applied a scale that defined the

TURKEY PLUNGES INTO ANTARCTIC WATERS

Şahika Ercümen, a record-setting free-diver—she uses no air tanks—heads for the surface on 4 February, off Antarctica's South Shetland Islands. Part of a research team from Turkey that journeyed to Antarctica to carry out 30 days of studies, she expects to observe whales and seals on her dives. Turkey holds an observer status under the Antarctic Treaty, which reserves the continent for research and other peaceful uses.

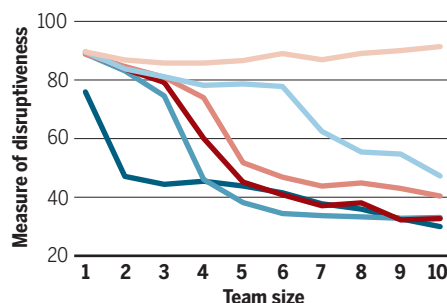


work as developmental if it was cited along with previous, similar research, and disruptive if it wasn't. Among highly cited research papers, those with one or two authors are three times more likely to be highly disruptive than papers with eight or more. The trend of greater disruption with smaller team size persists across time, journals, and most disciplines. Even so, large teams were more likely than small

Small teams shake it up

Articles were measured as "disruptive" based on patterns of citations to them.

Physical sciences Medicine Social sciences
Environmental and earth sciences Chemistry
Computer and information technology



ones to publish highly cited papers. Both kinds of teams are important to science and innovation, the authors conclude.

Men detail abuse by researcher

MISCONDUCT | Allegations of sexual abuse by a former university professor who studied growth problems in children were detailed last week after BuzzFeed News published graphic interviews with five male victims. It also reported that the journal *Radiology* removed from its website a 1965 article by the researcher, which contained photographs of naked boys. The alleged abuse involved Reginald Archibald, an endocrinologist at Rockefeller University in New York City from 1948 to 1982 who died in 2007. In October 2018, Rockefeller said Archibald had "engaged in certain inappropriate conduct during patient examinations" and that the university and its hospital "deeply regret" any "pain and suffering" to former patients. Although the statute of limitations for civil suits in New York in such cases has passed, Governor Andrew Cuomo said he will sign a bill passed by the legislature last month that would

make an exception for child victims. At least 150 people are considering such lawsuits, BuzzFeed reported.

Chinese transplants questioned

BIOETHICS | More than 440 research articles that used outcomes from at least 85,000 organ transplant operations in China should be retracted because the organs may have been harvested from executed prisoners, the authors of a new study say. International ethics standards ban publication of research that studied samples from executed prisoners. To check compliance, a group led by Wendy Rogers, an ethicist at Macquarie University in Sydney, Australia, examined peer-reviewed papers involving transplants done before January 2015, when Chinese authorities banned the use of organs from prisoners without consent. The analysis, appearing online in *BMJ Open* on 5 February, reports that 99% of the studies failed to specify whether the people from whom the organs were taken had given consent and 92% failed to state whether they came from executed prisoners.

Europe destroys GM crop

BIOTECHNOLOGY | Farmers in France and Germany were digging up nearly 10,000 hectares of fields planted with oilseed rape last week, after the discovery that a small fraction of the seeds—estimated to be less than 0.005%—were of a genetically modified (GM) variety not approved for planting in the European Union. Bayer, which distributed the seeds, recalled them. It is not known how the GM seeds, which are herbicide-tolerant and grown in North America, ended up mixed with non-GM seed that was grown in Argentina, which does not plant GM rapeseed. Commingling of GM and non-GM seed is rare, and it is even more unusual that the seeds are accidentally planted, says Justus Wesseler, an agricultural economist at Wageningen University in the Netherlands. Destruction of the crops, which could cost Bayer about \$21 million in compensation to farmers, could have been avoided if EU regulators

had set an acceptable threshold for trace amounts of unapproved GM seeds, he adds.

Rosalind to explore Mars

PLANETARY SCIENCE | The European-Russian ExoMars rover heading to the Red Planet next year has been given a new name: Rosalind Franklin, after the U.K. scientist who made key, belatedly recognized contributions that helped unravel the structure of DNA. The European Space Agency in Paris announced the name last week, after the public offered up 36,000 suggestions. In the 1950s, Franklin carried out x-ray diffraction of the DNA molecule that aided James Watson and Francis Crick in figuring out its structure. She died of ovarian cancer in 1958, at age 37, shortly before Watson, Crick, and her colleague Maurice Wilkins received a Nobel Prize for the work. Rosalind the rover will arrive at Mars in 2021. It will use a drill to study environments that once, or still could, support life.

U.S. teens' use of nicotine soars

PUBLIC HEALTH | Many more U.S. youths inhaled e-cigarettes in 2018, entirely driving a jump in their use of tobacco products and erasing reductions made in recent years, the U.S. Centers for Disease Control and Prevention reported on 11 February. The number who consumed other tobacco products stayed level. In 2018, 4.9 million middle and high school students reported using a tobacco product in the past 30 days, up about a third since 2017. Teens who use e-cigarettes are more likely to begin to use cigarettes and other tobacco products, which lead to preventable causes of disease and death. The U.S. Food and Drug Administration, which last year pressured manufacturers and sellers of e-cigarettes to curb purchases by teens, is planning additional regulatory steps.

Dingell left mark on science

SCIENCE POLICY | John Dingell, who died last week at age 92, served a record 59 years in the U.S. House of Representatives. He was known for his work on civil rights, health care, and environmental issues. But many scientists remember Dingell for his aggressive efforts to expose research misconduct and wasteful science spending. A “Dingell-gram” summoning a researcher or administrator to appear before his energy and commerce committee was a prelude to hours—or even days—of blunt questions from the Michigan Democrat. One late-1980s probe of alleged fraud in federally funded studies helped push biologist David Baltimore, a Nobel laureate, to retract his authorship of a high-profile paper. Dingell's work helped make addressing misconduct a major issue in the scientific community, and his template for such investigations remains in use today.

Supplements draw scrutiny

REGULATION | The U.S. Food and Drug Administration (FDA) said on 11 February it plans to revise how it oversees the dietary supplement industry. FDA Commissioner Scott Gottlieb voiced concern that a growing number of products are adulterated, or purport to treat or cure disease—a claim not permitted for such products, which do not go through the agency's premarket approval process. Last week, FDA issued 12 warning letters to supplementmakers for such misbranding. A newly formed public-private partnership will evaluate new research tools for assessing supplements' safety and effectiveness.

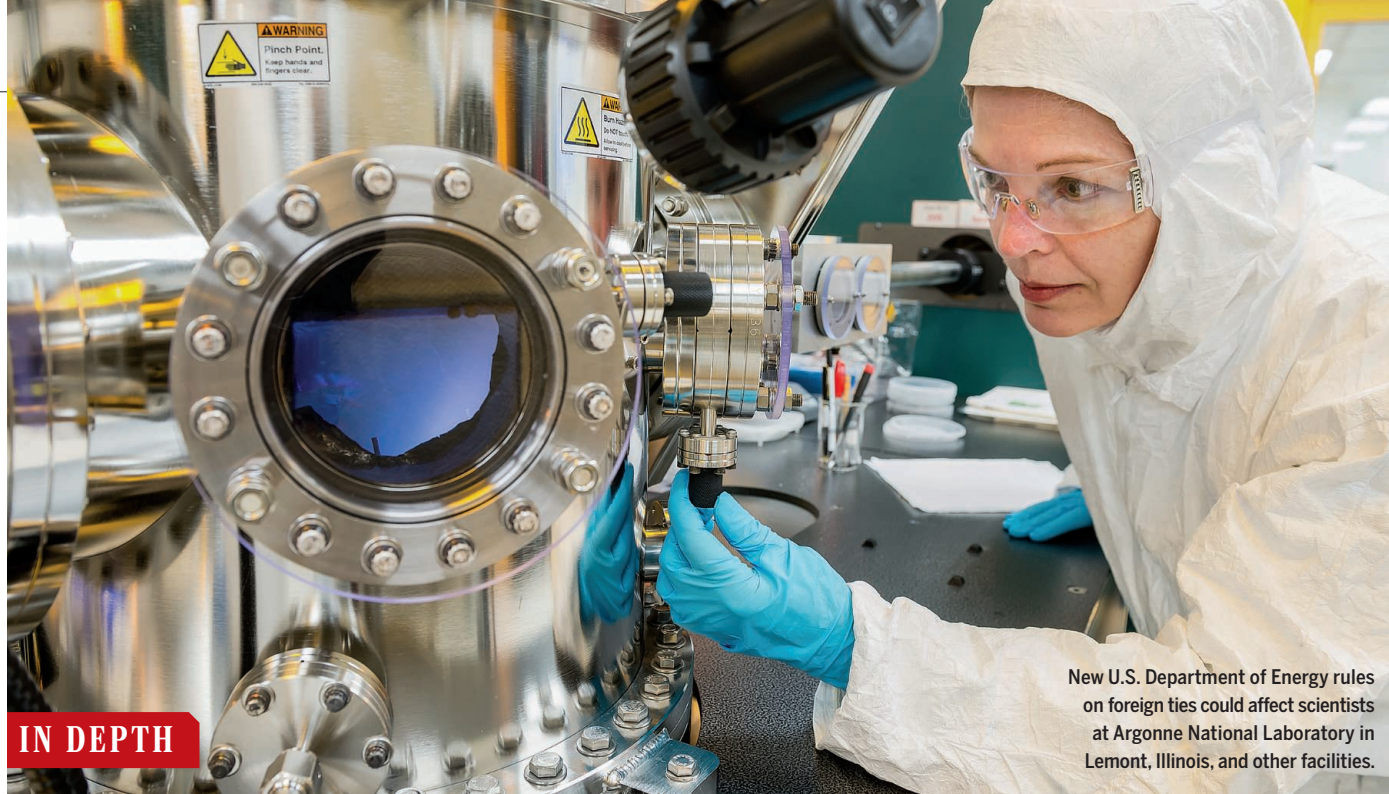


PLANETARY SCIENCE

The moon's far side, with home beyond

The far side of the moon, with Earth in the background, looms in this photo taken 4 February by a simple camera, built by students, on board the Chinese DSLWP-B/Longjiang-2 satellite. China placed it in orbit last year to conduct radio astronomy and then successfully deployed a lander on the far side on 3 January. The image was transmitted to the amateur-operated Dwingeloo radio telescope in the Netherlands.

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IN DEPTH

New U.S. Department of Energy rules on foreign ties could affect scientists at Argonne National Laboratory in Lemont, Illinois, and other facilities.

U.S. NATIONAL SECURITY

DOE to limit foreign research collaborations

Recent memos describe crackdown on grantees and on lab scientists with foreign ties

By Jeffrey Mervis

The U.S. Department of Energy (DOE) in Washington, D.C., has drafted new restrictions on research collaborations between the scientists it funds and certain foreign governments suspected of trying to steal sensitive technologies. DOE officials are still debating the scope of the new rules, which cover researchers at DOE's 17 national laboratories, as well as grantees. But some scientists are worried DOE is overreacting to growing concerns about scientific espionage and the theft of intellectual property.

The new policies, described in two recent memos to lab directors and other department officials from DOE Deputy Secretary Dan Brouillette, would prohibit DOE-funded researchers working in certain "emerging research areas and technologies" from collaborating with colleagues from "sensitive" countries. A 14 December 2018 memo says DOE-funded scientists working in those fields "will be generally prohibited" from traveling to those countries, although it does not identify either the nations or the emerging fields.

A 31 January memo, first reported by *The Wall Street Journal*, would bar researchers from participating in so-called talent

recruitment programs, which typically involve U.S. scientists spending at least some time abroad. Many countries sponsor such programs, but China's decade-old Thousand Talents program has attracted the most scrutiny from U.S. officials, both because it involves thousands of scientists and because a handful of participants have been prosecuted by the U.S. government for espionage.

DOE security policies historically have focused on protecting nuclear secrets, says Paul Dabbar, DOE's undersecretary for science, who oversees the national labs and its extramural research program. But he says that approach does not address newer technologies, such as quantum information science and genomics, which could also alter the balance of power among nations.

DOE "has done a very good job ... of keeping the peace around highly sensitive technologies ... and has made certain that certain types of R&D are held tightly within the lab context," he says. "But we're dealing with a new reality."

Dabbar did not quantify how many researchers might be affected by the new policies. But he says DOE grantees represent "an extremely narrow segment" of the overall U.S. scientific community, and

that scientists with other sources of funding will still have the chance to collaborate with their peers around the world. "We're not saying that universities can't take money from these countries; that is their decision," he explains. But academic researchers who maintain foreign collaborations that DOE deems risky would no longer be able to compete for department grants.

The ban on participating in foreign-talent programs similarly targets a relative handful of scientists, he adds. "If you're working for [DOE], and taking taxpayer dollars, we don't want you to work for [foreign countries] at the same time," he explains.

DOE will allow exemptions for "government to government" collaborations, the December memo notes. That suggests the policy shouldn't affect major international projects such as the ITER fusion experiment under construction near Cadarache in France, or the Long-Baseline Neutrino Facility being developed at Fermi National Accelerator Laboratory in Batavia, Illinois. The memo says DOE will also allow smaller collaborations if researchers can provide officials with "a clear description of why this agreement benefits the United States."

Dabbar says the policy is more flexible than the memos indicate. Although the December memo declares that DOE grantees would be barred “from using U.S. tax dollars to conduct international research collaborations or support sensitive country foreign nationals” working on certain kinds of research, Dabbar told *Science*, “That is not the case.”

Asked whether the memo had incorrectly characterized the pending policy, Dabbar replied, “Yeah, yeah. The reality is that the implementation of specifics has not been finalized, and that there is flexibility for exceptions, even within a particular [research] area.”

Exactly which nations will appear on the so-called risk matrix the department is preparing is unclear. DOE already identifies some 30 countries as “sensitive” for travel and security purposes. The list includes U.S. allies such as Israel and India, but also nations deemed “terrorist” states, including North Korea and Iran. Dabbar suggested the risk matrix might include fewer countries and that the number of proscribed foreign-talent recruitment programs could be even smaller.

“I can tell you some of the countries that aren’t on [the list],” Dabbar added, citing Canada, Germany, Australia, and the United Kingdom. “This is not targeting countries that we work with all the time and who are not looking to appropriate our technology,” he says. He also noted that the Department of Defense has identified just four countries—China, Russia, Iran, and North Korea—as especially sensitive competitors.

Last week, Dabbar met with research administrators at several major universities to outline and answer questions about the new policies. “We don’t want to implement this without engaging the universities,” he says.

One university lobbyist who requested anonymity admitted that some institutions are not aware of every international collaboration involving faculty members, and said full disclosure is essential. But another university lobbyist noted that the new policies appear to clash with two core academic principles: giving students unfettered access to research opportunities and avoiding distinctions based on national origin. Barring professors from DOE grants would violate the first principle, and preventing faculty from working with scientists from a particular country would undermine the second.

University leaders and lab directors are now waiting anxiously to learn more from DOE. One lobbyist says: “This is a pretty big deal.” ■

INFECTIOUS DISEASES

Controversial flu studies can resume, U.S. panel says

Critics complain of lack of transparency in decision that allows efforts to create potentially risky virus strains

By Jocelyn Kaiser

Controversial lab studies that modify bird flu viruses in ways that could make them more risky to humans will soon resume after being on hold for more than 4 years. *Science* has learned that last year, a U.S. government review panel quietly approved two projects previously considered so dangerous that federal officials had imposed an unusual moratorium on the research.



Yoshihiro Kawaoka (left) and Ron Fouchier (right) in 2012, after their flu research sparked controversy.

One project already has funding from the National Institutes of Health’s (NIH’s) National Institute of Allergy and Infectious Diseases (NIAID) and will start in a few weeks; the other is awaiting funding.

The outcome marks the latest twist in a nearly decadelong debate over the risks and benefits of studies that aim to make pathogens more potent or more likely to spread in order to better understand them and prepare defenses. And it represents the first test of a new federal review process intended to ensure that government funding flows to such studies only when they are scientifically and ethically justifiable and done under strict safety rules.

The researcher leading one of the approved studies says he’s looking forward to resuming the work. “We are glad the

United States government weighed the risks and benefits. ... We know that [the research] does carry risks. We also believe it is important work to protect human health,” says Yoshihiro Kawaoka of the University of Wisconsin in Madison and the University of Tokyo. He learned on 10 January that NIH had funded his proposal. The other group that won approval, but has not yet received funding, is led by Ron Fouchier at Erasmus Medical Center in Rotterdam, the Netherlands.

Some other researchers are sharply critical of the approvals, saying the review lacked transparency. After a long public discussion to develop new standards that “consumed countless weeks and months of time for many scientists, we are now being asked to trust a completely opaque process,” says Harvard University epidemiologist Marc Lipsitch, who helped lead a push to require the reviews.

In 2011, Fouchier and Kawaoka alarmed the world by revealing they had each modified the H5N1 influenza virus, which kills birds, so that it could spread between ferrets, a model for studying flu in people. Advocates say such gain of function (GOF) studies help experts plan for pandemics. But the work also raised fears that the souped-up virus could jump to humans. Critics warned it could escape from a lab or be weaponized. After a debate about whether the studies should be published (they were), and a voluntary moratorium on such experiments, the two labs resumed the work in 2013 under new U.S. rules.

In 2014, however, controversy flared anew after these two labs and others published GOF studies for H5N1 and other potent bird flu strains and U.S. government laboratories working with pathogens had several accidents. In October 2014, U.S. officials announced an unprecedented “pause” on funding for 18 GOF studies of viruses that cause influenza, Middle East respiratory syndrome, or severe acute respiratory syndrome. (They later allowed about half to continue.)

In December 2017, U.S. officials lifted the funding pause, but many studies remained on hold. To resume, they had to comply with

a new policy for evaluating proposed studies involving “enhanced potential pandemic pathogens.” NIH invited new GOF proposals, which would be reviewed by a panel of experts from the Department of Health and Human Services (HHS) in Washington, D.C., and other federal agencies.

Kawaoka and Fouchier, the only scientists so far to submit proposals, essentially plan to continue studies interrupted by the funding pause. The HHS panel reviewed those proposals last summer, and a department spokesperson said it approved them late last year after the researchers accepted recommendations to revise their risk-benefit analyses, some safety measures, and communications plans.

Kawaoka wants to identify mutations in H5N1 that could allow ferrets to infect each other via respiratory droplets. His grant includes requirements that he immediately notify NIAID if he identifies an H5N1 strain that is both able to spread via droplets in ferrets and is highly pathogenic, or if he develops a dangerous strain that is resistant to antiviral drugs.

Fouchier’s proposed projects include identifying molecular changes that make flu viruses more virulent. As with Kawaoka, the panel did not ask him to remove any experiments. But Fouchier says it suggested his team clarify how it will monitor workers for exposure and justify the flu strains it wants to work with, which include another bird virus, H7N9.

HHS “is committed to being as transparent as possible,” a spokesperson said, and an advisory panel will be publicly examining the review process “once a sufficient number of reviews have been conducted.” But the department cannot release the specific reviews because they contain confidential or proprietary information. That lack of openness is “indefensible,” says microbiologist Richard Ebright of Rutgers University in Piscataway, New Jersey. “Details regarding the decision to approve and fund this work should be made transparent,” adds Thomas Inglesby, director of the Center for Health Security of the Johns Hopkins Bloomberg School of Public Health in Baltimore, Maryland. The critics say the HHS panel should at least explain why it thought the same questions could not be answered using safer alternative methods.

One researcher who has sympathized with both sides in the debate finds the safety conditions imposed on Kawaoka reassuring. “That list ... makes a lot of sense,” says virologist Michael Imperiale of the University of Michigan in Ann Arbor. “At this point I’m willing to trust the system.” ■

A nurse gives a child a measles shot at City Children’s Hospital in Odessa, Ukraine, in October 2018.



INFECTIOUS DISEASE

Measles epidemic in Ukraine drove troubling European year

War and distrust of vaccines seeded ongoing outbreak

By Meredith Wadman

Measles cases more than tripled across Europe in 2018, and one country drove much of the surge: Ukraine. Nearly 83,000 cases of measles were reported in the World Health Organization’s (WHO’s) European Region in 2018, compared with some 25,500 in 2017, WHO, headquartered in Geneva, Switzerland, announced last week. Ukraine had more than 54,000 cases in 2018, its government says. Last year, 16 Ukrainians died of the extremely contagious viral disease, which is easily prevented with a vaccine.

“The current epidemic is the most massive in the entire postvaccine period,” says Nataliya Vynnyk, a pediatric infectious disease specialist at Children’s Clinical Hospital in Kyiv. With more than 15,000 cases and seven deaths between 28 December 2018 and 1 February, according to the country’s Ministry of Health (MOH), the epidemic continues to worsen.

Ukraine’s government is taking action. “It’s egregious to have people have measles in the 21st century in a European country,” says Ulana Suprun, a physician who has been Ukraine’s acting minister of health since August 2016. She blames a decade of corrup-

tion, war, a lack of political commitment to vaccination, and antivaccine sentiment.

Measles is spread by respiratory droplets. Most people recover, but the disease can cause sometimes-fatal complications including pneumonia and inflammation of the brain. Typically, children are vaccinated around their first birthday and again before starting school. According to WHO, 95% of children need to be fully vaccinated to stop the disease from spreading.

Elsewhere in Europe, vaccine skepticism has given the virus an opening. Cases in Greece doubled from 2017 to 2018; cases in France grew nearly sixfold. Meanwhile, the United States logged 372 cases last year. An outbreak in Washington this year has resulted in 53 confirmed cases as of 11 February, nearly all in unvaccinated children, pushing the U.S. year-to-date tally above 100.

In the past decade, vaccine refusal has also played a big role in Ukraine. In 2008, a day after receiving the measles vaccine, a 17-year-old died—from an unrelated cause, according to WHO and the United Nations International Children’s Emergency Fund (UNICEF). His death led to a huge loss of confidence among parents: Vaccination rates plunged from 97% of 1-year-olds in 2007 to 56% in 2010. Coverage then slowly

improved, reaching 79% in 2012 and 2013.

But in 2014, then-President Viktor Yanukovich was ousted after violent protests, Russia annexed Crimea, and armed conflict broke out in eastern Ukraine. Paralyzed, the government failed to order measles vaccine until late 2015. Because of shortages, in 2016, Ukraine vaccinated only 42% of its infants. And that year, just 31% of 6-year-olds received the recommended second measles shot—one of the lowest rates in the world.

“If you have a high rate of unvaccinated children, you will face this kind of outbreak,” says Vusala Allahverdiyeva, a physician who is a technical officer in WHO’s Kyiv office.

There are other problems, too. Authorities have grappled with basic challenges. Investigating an outbreak of 90 cases in children in a mountainous region in western Ukraine in 2018, MOH found the children had been vaccinated at a clinic that experienced frequent power outages and had no generator. (The vaccine must be stored at less than 8°C to be effective.)

A faulty vaccine may also have played a role. According to MOH, more than 20,000 of those infected in 2018 were adults who would have been vaccinated decades ago with a Russian vaccine that was dropped in 2001. The ministry is trying to identify whether that vaccine was less effective in some years, with an eye to revaccinating those who received it.

MOH has been striving to catch up. It is now procuring the vaccine more cheaply through UNICEF; sending mobile vaccination brigades from school to school in Lviv, a hard-hit area; and preparing to provide cash incentives to Ukraine’s underpaid physicians to vaccinate all children in their practices. In 2017, the country’s coverage rebounded to 93% of infants and 91% of 6-year-olds.

But because of Ukraine’s large pool of unvaccinated or undervaccinated people, the epidemic roars on. “We need to start thinking outside the box,” Suprun says. For instance, UNICEF is working with the government on a campaign urging grandparents, who often care for young children in Ukraine, to get their charges vaccinated.

The Ukrainian outbreak caps a year in which measles cases surged around the globe. As of mid-January, WHO had received reports of more than 229,000 measles cases in 2018. The global number is expected to rise by the time it is finalized in June, but it’s already a 32% increase from 2017.

“The root cause of the measles outbreaks ... is a failure to adequately vaccinate,” says Katrina Kretsinger, the lead measles expert at WHO headquarters. “Many people in many countries remain susceptible, and large pockets of susceptible persons can lead to large outbreaks.” ■

EVOLUTION

How rabbits escaped a deadly virus—at least for now

Same genetic changes found in rabbits on two continents

By Elizabeth Pennisi

Researchers have written another chapter in the textbook case of an arms race between a host and its pathogen. The main characters in this 70-year seesaw drama are the voracious European rabbit (*Oryctolagus cuniculus*) and a virus deliberately released in France and Australia to kill off the rabbits and protect fields and pastures. Working with museum specimens collected decades ago, a team has discovered that rabbits on two continents evolved the same genetic changes to beat back the virus—before the virus itself changed and regained the upper hand.

The find is a striking example of how evolution sometimes repeats itself, and it may hold clues to how human immune systems respond—or don’t—to pathogens. The rabbit work, published online this week in *Science*, “provides key new information on one of the greatest stories in evolution,” says Edward Holmes, an evolutionary biologist at the University of Sydney in Australia who studies the biocontrol virus.

In Australia, a few dozen European rabbits introduced in the mid-1800s for hunters did what the animals famously do. They multiplied until hundreds of millions were chowing down on crops. So, in 1950, after a smallpoxlike virus found in South American rabbits turned out to kill the European rabbit, Australian authorities released the virus into the wild, cutting the rabbit population by 99%. A few years later, the virus, called myxoma, was released in France and eventually spread to the United Kingdom.

The result was “an opportunity to trace host-pathogen arms races right in front of our eyes,” says Jia Liu, a biologist at the University of Arkansas for Medical Sciences in Little Rock. Within a decade, rabbit numbers were on the rise again as some evolved resistance to this deadly infection and the virus itself became less deadly.

To understand the rabbit’s adaptations,

Joel Alves, now an evolutionary biologist at the University of Oxford in the United Kingdom, evolutionary geneticist Francis Jiggins at the University of Cambridge in the United Kingdom, and colleagues tracked down specimens of U.K., Australian, and French *O. cuniculus* collected by museums prior to the virus’s introduction. They sequenced all the genes and other DNA that might influence the body’s immune defenses and compared the results with sequences from modern rabbits living in the same places. The comparisons revealed changes in many genes, usually a shift in the frequency of particular versions, or alleles, of a gene. Strikingly, half of the changes were shared by the rabbits in all three countries—evidence of parallel evolution.

One allele shift affected the rabbits’ interferon, a protein released by immune cells that sounds the alarm about a viral attack and helps trigger an immune response. Compared with preinfection rabbits, modern rabbits make an interferon that is better at responding to the biocontrol virus, the researchers found when they added different versions of the protein to rabbit cell lines.

The virus has not stood still. In 2017, Holmes and his colleagues reported that in the 1970s the virus developed a greater ability to suppress the rabbit’s immune responses. That change, as well as the natural emergence of another rabbit-killing virus, has caused populations to decline again. But in contrast to the parallel evolution in rabbits, myxoma viruses in the various locations took different genetic paths to regaining potency.

Andrew Read, an evolutionary microbiologist at Pennsylvania State University in State College, suggests the viral counter-attack “is a cautionary tale” for researchers aiming to take charge of the evolutionary arms race by introducing biocontrol agents or making crops or livestock more resistant to disease. “One should be careful about evolution and counterevolution,” he says. “The rabbit hasn’t won.” ■



The European rabbit devastated crops and pastures in Australia and elsewhere.



The Indian government in 2017 decided to fund research to validate claims that panchagavya, a mixture that includes cow urine and dung, has therapeutic value.

SCIENCE AND RELIGION

In India, Hindu pride boosts pseudoscience

Claims of great technological achievements in ancient times trigger ridicule and concern

By **Sanjay Kumar**, in New Delhi

The most widely discussed talk at the Indian Science Congress, a government-funded annual jamboree held in Jalandhar in January, wasn't about space exploration or information technology, areas in which India has made rapid progress. Instead, the talk celebrated a story in the Hindu epic *Mahabharata* about a woman who gave birth to 100 children, citing it as evidence that India's ancient Hindu civilization had developed advanced reproductive technologies. Just as surprising as the claim was the distinguished pedigree of the scientist who made it: chemist G. Nageshwar Rao, vice-chancellor of Andhra University in Visakhapatnam. "Stem cell research was done in this country thousands of years ago," Rao said.

His talk was widely met with ridicule. But Rao is hardly the only Indian scientist to make such claims. In recent years, "experts" have said ancient Indians had spacecraft, the internet, and nuclear weapons—long before Western science came on the scene.

Such claims and other forms of pseudoscience rooted in Hindu nationalism have been on the rise since Prime Minister Narendra Modi came to power in 2014. They're not just an embarrassment, some researchers say, but a threat to science and education that stifles critical thinking and could hamper India's development. "Modi has initiated what may be called 'Project Assault on Scientific Rationality,'" says Gauhar Raza, former chief scientist at the Council of Scientific and Industrial

Research (CSIR) here, a conglomerate of almost 40 national labs. "A religio-mythical culture is being propagated in the country's scientific institutions aggressively."

Some blame the rapid rise at least in part on Vijnana Bharati (VIBHA), the science wing of Rashtriya Swayamsewak Sangh (RSS), a massive conservative movement that aims to turn India into a Hindu nation and is the ideological parent of Modi's Bharatiya Janata Party. VIBHA aims to educate the masses about science and technology and harness research to stimulate India's development, but it also promotes "Swadeshi" (indigenous) science and tries to connect modern science to traditional knowledge and Hindu spirituality.

VIBHA receives generous government funding and is active in 23 of India's 29 states, organizing huge science fairs and other events; it has 20,000 so-called "team members" to spread its ideas and 100,000 volunteers—including many in the highest echelons of Indian science.

VIBHA's advisory board includes Vijay Kumar Saraswat, former head of Indian defense research and now chancellor of Jawaharlal Nehru University here. The former chairs of India's Space Commission and its Atomic Energy Commission are VIBHA "patrons." Structural biologist Shekhar Mande, director-general of CSIR, is VIBHA's vice president.

Saraswat—who says he firmly believes in the power of gemstones to influence well-being and destiny—is proud of the achievements of ancient Hindu science: "We should rediscover Indian systems which existed thousands of years back," he says. Mande

shares that pride. "We are a race which is not inferior to any other race in the world," he says. "Great things have happened in this part of the world." Mande insists that VIBHA is not antiscientific, however: "We want to tell people you have to be rational in your life and not believe in irrational myths." He does not see a rise of pseudoscience in the past 4 years—"We have always had that"—and says part of the problem is that the press is now paying more attention to the occasional bizarre claim. "If journalists don't report it, actually that would be perfect," he says.

But others say there is little doubt that pseudoscience is on the rise—even at the highest levels of government. Modi, who was an RSS *pracharak*, or propagandist, for 12 years, claimed in 2014 that the transplantation of the elephant head of the god Ganesha to a human—a tale told in ancient epics—was a great achievement of Indian surgery millennia ago, and has made claims about stem cells similar to Rao's. At last year's Indian Science Congress, science minister Harsh Vardhan, a medical doctor and RSS member, said, incorrectly, that physicist Stephen Hawking had stated that the Vedas include theories superior to Albert Einstein's equation $E=mc^2$. "It's one thing for a crackpot to say something like that, but it's a very bad example for people in authority to do so. It is deplorable," Venki Ramakrishnan, the Indian-born president of the Royal Society in London and a 2009 Nobel laureate in chemistry, tells *Science*. (Vardhan has declined to explain his statement so far and did not re-

spond to an interview request from *Science*.)

Critics say pseudoscience is creeping into science funding and education. In 2017, Vardhan decided to fund research at the prestigious Indian Institute of Technology here to validate claims that panchagavya, a concoction that includes cow urine and dung, is a remedy for a wide array of ailments—a notion many scientists dismiss. And in January 2018, higher education minister Satya Pal Singh dismissed Charles Darwin's evolution theory and threatened to remove it from school and college curricula. "Nobody, including our ancestors, in written or oral [texts], has said that they ever saw an ape turning into a human being," Singh said.

Those remarks triggered a storm of protest; in a rare display of unity, India's three premier science academies said removing evolution from school curricula, or diluting it with "non-scientific explanations or myths," would be "a retrograde step." In other instances, too, scientists are pushing back against the growing tide of pseudoscience. But doing so can be dangerous. In the past 5 years, four prominent fighters against superstition and pseudoscientific ideas and practices have been murdered, including Narendra Dabholkar, a physician, and M. M. Kalburgi, former vice-chancellor of Kannada University in Hampi. Ongoing police investigations have linked their killers to Hindu fundamentalist organizations.

Some Indian scientists may be susceptible to nonscientific beliefs because they view science as a 9-to-5 job, says Ashok Sahni, a renowned paleontologist and emeritus professor at Panjab University in Chandigarh. "Their religious beliefs don't dovetail with science," he says, and outside working hours those beliefs may hold sway. A tradition of deference to teachers and older persons may also play a role, he adds. "Freedom to question authority, to question writings, that's [an] intrinsic part of science," Ramakrishnan adds. Rather than focusing on the past, India should focus on its scientific future, he says—and drastically hike its research funding.

The grip of Hindu nationalism on Indian society is about to be tested. Two dozen opposition parties have joined forces against Modi for elections that will be held before the end of May. A loss by Modi would bring "some change," says Prabir Purkayastha, vice president of the All India People's Science Network in Madurai, a liberal science advocacy movement with some 400,000 members across the country that opposes VIBHA's ideology. But the tide of pseudoscience may not retreat quickly, he says. "I don't think this battle is going to die down soon, because institutions have been weakened and infected." ■

Sanjay Kumar is a journalist in New Delhi.

PUBLIC HEALTH

AIDS push gets mixed reviews

Trump's initiative to "end AIDS" promises money, focus

By Jon Cohen

When advocacy groups heard that President Donald Trump's State of the Union address last week would include a call for ending the AIDS epidemic in the United States by 2030, some weighed in with guffaws. One, the nonprofit AIDS Coalition to Unleash Power New York in New York City, published a list of ways it says the Trump administration has "further marginalized people living with HIV." But many HIV/AIDS researchers and some advocates had a more measured, even enthusiastic, reaction to the possibility that Trump wants to join an existing ambitious campaign and champion a cause he thus far has not embraced.

"Together, we will defeat AIDS in America and beyond," Trump said in the 5 February speech. His proposal—which officials fleshed out later—calls for concentrating on the places with the highest rates of new HIV diagnoses. According to U.S. Centers for Disease Control and Prevention (CDC) data from 2016, 48 counties in 19 states, plus Washington, D.C., and one municipality in Puerto Rico, account for more than half of the nearly 40,000 new HIV diagnoses each year.

Brett Giroir, deputy director of the Department of Health and Human Services (HHS) in Washington, D.C., says Trump's budget request, expected next month, will ask Congress for new funding for the initiative, although he would not say how much. "We are very confident we will have sufficient resources," he says.

"I'm really excited that this may lead to something," Carlos del Rio, an epidemiologist and leading HIV/AIDS clinician at Emory University's Rollins School of Public Health in Atlanta, told *Science*. "It's the last thing I would have expected coming out of Trump."

The initiative took shape last summer, when Robert Redfield, head of CDC in Atlanta and a longtime HIV/AIDS clinician and researcher, met with Anthony Fauci, who heads the U.S. National Institute of Allergy and Infectious Diseases in Bethesda, Maryland. They shared ideas about how to end the country's HIV/AIDS epidemic, then took their proposal to HHS head Alex Azar. "Alex really, really liked it," Fauci says. "He said,

'I think we can bring this to the president.'"

The Trump administration aims to reduce new infections by 75% in 5 years and 90% in 10. The plan would target the most affected populations, among them men who have sex with men and people who inject drugs. It would promote condom use, clean needles and syringes, aggressive testing, and immediate treatment for those who are positive. It would also help infected people stay on treatment, which can both stave off AIDS and make them less infectious. The million or so people deemed at high risk of infection would be encouraged to take anti-HIV drugs themselves, a proven prevention strategy known as pre-exposure prophylaxis (PrEP).

"I don't know that there's a big gap in the past strategy," says Jeffrey Crowley, a public health specialist who directed the White House Office of National AIDS Policy during then-President Barack Obama's administration and now works at the O'Neill Institute for National and Global Health Law at Georgetown University Law Center in Washington, D.C. "Maybe there's a more intentional way to do things and [the Trump administration] might identify new resources."

Some say current policies are an obstacle. Mark Harrington, who heads the Treatment Action Group in New York City and helped launch New York's ending AIDS campaign, cites policies he says have undermined the rights of groups disproportionately affected by HIV, including transgender people, communities of color, and undocumented immigrants. There are "major obstacles to getting essential HIV prevention tools into the hands of the people who need them," he says.

Epidemiologist Chris Beyrer of the Johns Hopkins University Bloomberg School of Public Health in Baltimore, Maryland, also blames Republicans who opposed the expansion of Medicaid. "HIV is worsening in the American South because low-income and working poor African-Americans are being excluded from health care access."

"We might be able to end AIDS as a public health threat in the U.S. by 2030, but we won't get there with testing and condoms," Beyrer says. "We have to do better with [the] provision of health care, drug treatment, anti-retroviral treatment, and PrEP to those who need it most." ■

"We are very confident we will have sufficient resources."

Brett Giroir, HHS

Metric prefixes sought for extreme numbers

“Ronna” and “quecca” would help computer scientists keep pace with big data

By David Adam

Fresh from redefining the kilogram and other fundamental measures, the guardians of the metric system have set their sights on another upgrade: new prefixes for outrageously large and small numbers.

A proposal lodged with the International Bureau of Weights and Measures (BIPM) in Paris recommends new names—ronna and quecca—as prefixes for 10^{27} and 10^{30} , respectively. They would be joined by their microscopic counterparts, ronto for 10^{-27} , and quecto for 10^{-30} . If approved, the new terms could be formally introduced in 2022. They would be the first prefixes added since 1991.

The planned update responds to the massive growth in global data storage, which by the early 2030s is forecast to reach 1 yottabyte (10^{24})—the top of the existing scale. Without new prefixes, computer scientists will have no way to officially talk about what comes next. At the other end of the scale, quantum physicists have measured atomic forces as small as 42 yoctonewtons. Much smaller and they run out of metrological road.

“Where there is a need that is not met, there is also a risk that unofficial units can take hold and that can cause confusion,” says Richard Brown, head of metrology at the National Physical Laboratory near London, who came up with the new names. He says unofficial terms beyond yotta, including brontobyte and geobyte, are already becoming popular. Although mathematicians sometimes use the prefix googol (10^{100}), a name coined a century ago by a 9-year-old girl, it, too, is unofficial.

Brown prefers to follow tradition. The new prefixes should relate etymologically to nine and 10, to represent the ninth and 10th powers of 10^3 . He also wanted to continue the reverse alphabetical trend set by zetta

and yotta, but needed to avoid letters such as X, W, and V that could be confused with other terms. And so, drawing from the Latin and Greek words for nine (*novem, ennea*) and 10 (*decem, deka*), with some poetic license to make the terms more easily pronounced, he came up with ronna, quecca, ronto, and quecto. “It’s supposed to be a conversation starter,” says Brown, who published his proposal last month in the journal *Measurement*.

The terms are due to be discussed at the October meeting of BIPM’s Consultative Committee for Units. If the committee approves the idea, it could make a formal recommendation to BIPM. The organization’s general conference, which includes government representatives and is due to next meet in 2022, would have the final vote—as

it did late last year when it approved a new definition of the kilogram based on fundamental physical constants (*Science*, 9 November 2018, p. 625).

It’s too early to say whether the prefixes will be adopted, says Estefanía de Mirandés, executive secretary of the units committee and a physicist with BIPM. “It would be premature to mention a possible outcome of the discussion,” she wrote in an email.

Other proposals to extend the measurement scale have fizzled. In 2010, a physics student in California suggested “hella” as a prefix for 10^{27} , and thousands of people signed an online petition in support. (Contrary to reports, the idea did not reach the BIPM units committee for formal discussion.) In 2008, an article in *The New York Times* on super-

computers referred to a xeraflop, and a 2015 paper on cosmic engineering used the symbols X, W, and V to describe the gargantuan energy levels, beyond the yotta scale, that could be seen if aliens turned a black hole into a particle accelerator. One prankster hacked a Wikipedia article in 2008 to introduce a new technical term for a computer that could attempt 10^{48} operations per second: a gonnaflop. It lasted 7 minutes before being deleted.

Ronna, quecca, and their partners could fare better. Emilio Prieto, who represents the Spanish Metrology Center in Madrid on the units committee, says he would vote for the names because they are simple and memorable. “Once people start using the wrong prefix names it is impossible to go back,” he says.

If those four are approved, Brown says, only a single good letter would remain that could be used on its own for 10^{33} and 10^{-33} in future: B (and b). Brown already has names at the ready: bundecca and bundecto, based on the Latin for 11, *undecim*. ■

David Adam is a journalist based near London.

A whole lotta yottas

Metrologists are proposing to extend metric prefixes beyond yotta and yocto. By the 2030s, computer data storage (pictured) may surpass 1 yottabyte. Scientists need numbers to describe this new regime.

PREFIX	SYMBOL	POWER
quecca	Q	10^{30}
ronna	R	10^{27}
yotta	Y	10^{24}
zetta	Z	10^{21}
exa	E	10^{18}
peta	P	10^{15}
tera	T	10^{12}
giga	G	10^9
mega	M	10^6
kilo	k	10^3
milli	m	10^{-3}
micro	μ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}
atto	a	10^{-18}
zepto	z	10^{-21}
yocto	y	10^{-24}
ronto	r	10^{-27}
quecto	q	10^{-30}





THE TWITTER WARRIOR

#MeToo provocateur BethAnn McLaughlin battles on behalf of women in STEM—but her own job is in peril

By **Meredith Wadman**

BethAnn McLaughlin has no time for James Watson, especially not when the 90-year-old geneticist is peering out from a photo on the wall of her guest room at Cold Spring Harbor Laboratory's Banbury Center.

"I don't need him staring at me when I'm trying to go to sleep," McLaughlin told a December 2018 gathering at the storied New York meeting center as she projected a photo of her redecorating job: She had hung a washcloth over the image of Watson, who co-discovered DNA's structure, directed the lab for decades—and is well-known for racist and sexist statements.

The washcloth image was part of McLaughlin's unconventional presentation—by turns sobering, hilarious, passionate, and profane—to two dozen experts who had gathered to wrestle with how to end gender discrimination in the biosciences. McLaughlin, a 51-year-old neuroscientist at Vanderbilt University Medical Center (VUMC) in Nashville, displayed the names of current members of the National Academy of Sciences (NAS) who have been sanctioned for sexual harassment. She urged other NAS members—several of whom sat in the room—to resign in protest, "as one does." She chided institutions for passing along "harassholes" to other universities. "The only other places that do this are the Catholic Church and the military," she said.

In the past 9 months, McLaughlin has exploded into view as the public face of the #MeToo movement in science, wielding her irreverent, sometimes wickedly funny Twitter presence, @McLNeuro, as part cudgel, part cheerleader's megaphone. In June 2018, she created a website, MeTooSTEM.com, where scores of women in science, technology, engineering, and math (STEM) have posted mostly anonymous, often harrowing tales of their own harassment. In just 2 days that month, she convinced the widely used website RateMyProfessors.com to remove its "red hot chili pepper"

rating for "hotness." And after launching an online petition, she succeeded last fall in spurring AAAS, which publishes *Science*, to adopt a policy allowing proven sexual harassers to be stripped of AAAS honors (*Science*, 21 September 2018, p. 1175).

"It's clear that she has a voice and that people are listening," says biologist Carol Greider of Johns Hopkins University School of Medicine in Baltimore, Maryland, and a co-organizer of the Banbury Center meeting. "She is really trying to change society," adds Carrie McAdams, a psychiatrist and neuroscientist at the University of Texas Southwestern Medical Center in Dallas, who sought out McLaughlin by phone last year to discuss how to report long-ago harassment.

In November 2018, McLaughlin shared the second annual \$250,000 Disobedience Award from the Massachusetts Institute of Technology's Media Lab in Cambridge for "ethical, nonviolent" civil disobedience. And, "impressed by the sheer force of her conviction" at the Banbury meeting, Erin O'Shea, president of the Howard Hughes Medical Institute in Chevy Chase, Maryland, later pledged the institute's financial support for a nonprofit, #MeTooSTEM, that McLaughlin is founding to support survivors of sexual harassment.

Anita Hill, a professor at Brandeis University in Waltham, Massachusetts, who in 1991 accused then-Supreme Court nominee Clarence Thomas of sexual harassment, emailed McLaughlin last summer thanking her for speaking out. Hill also recognized that McLaughlin would pay a price: "The impact on you and your career is not to be underestimated," Hill wrote.

Indeed, McLaughlin has made bitter enemies: Last fall, she says, she was anonymously FedExed a box of feces. And her scientific career is now on the line. Her tenure process was frozen for 17 months starting in 2015

while VUMC investigated allegations that she had posted anonymous, derogatory tweets about colleagues. The probe was spurred by complaints from a professor whom she had testified against in a sexual harassment investigation. VUMC closed the probe without disciplining McLaughlin, but in 2017 a faculty committee, having previously approved her tenure, unanimously reversed itself, according to university documents. Absent a last-minute reprieve, she will lose her job on 28 February, when her National Institutes of Health (NIH) grant expires.

McLaughlin says she has not been looking for other jobs and hopes to continue in science. She says she has seven manuscripts in development and recently submitted a new NIH grant application. But she is consumed with #MeTooSTEM efforts, from supporting individual survivors to meeting with NIH Director Francis Collins in Bethesda, Maryland, last week and addressing his new working group on sexual harassment. "I have a real strong belief that the [Vanderbilt] chancellor and the Board of Trust are going to do the right thing," she says. "If not now, when?"

MCLAUGHLIN GREW UP in Missouri and New Hampshire, exposed to both science and politics. Her mother was an elementary school teacher and her father an engineering graduate student. He died suddenly when McLaughlin was 8, and the struggling family plunged deeper into poverty.

Despite their difficulties, her mother found time to support women campaigning for city council. "A lot of our community was people she politicked with," McLaughlin says.

McLaughlin graduated from Skidmore College in Saratoga Springs, New York, and completed a Ph.D. in neuroscience at the University of Pennsylvania. She "was intense ... and intellectually deep," recalls her postdoc adviser Elias Aizenman of the University of Pittsburgh in Pennsylvania. "She liked to work long and hard hours. She had high expectations of her peers. And of herself."

She was first author on a 2003 *Proceedings of the National Academy of Sciences* paper that she and Aizenman initially had trouble placing, because it challenged dogma by finding that certain proteins linked to cell death also triggered a cell survival pathway in the brain. “I think I instilled in her to not be afraid to stick her neck out,” Aizenman says. “I admire her now for sticking her neck out in a different direction.”

In 2002, McLaughlin took a position as a research assistant professor at Vanderbilt’s School of Medicine and was promoted to tenure-track assistant professor in 2005. She focused on understanding how brain cells cope with oxygen deprivation during strokes and cardiac arrest, with an eye to finding therapies. She trained students and published steadily in respected journals. (In 2017, two instances of possible image duplication between her papers were flagged on the website PubPeer. McLaughlin acknowledged a mistake, apologized, and requested a correction in one case; a co-author explained the other.)

In 2005, she was key to landing a \$1.2 million private donation to launch an autism research institute at Vanderbilt’s Kennedy Center. She also founded VUMC’s Clinical Neuroscience Scholars Program in 2011, which links neuroscience graduate students with clinical experts, so students can see real-life manifestations of the conditions they study.

In 2015, McLaughlin helped launch a Vanderbilt-hosted blog, *Edge for Scholars*, that bills itself as a space for “gritty truths” about academic life. Blogging anonymously as “Fighting Squirrel,” she began to develop a public voice on issues she saw affecting female scientists, such as authorship inequity. Her posts soon became the site’s most popular, with more than 400,000 views to date.

“She was blogging about the larger MeTooSTEM movement before it had a hashtag,” says Katherine Hartmann, an associate dean at VUMC who recruited McLaughlin. “She’s our top blogger, top recruiter, top social media maven that makes it go.”

MCLAUGHLIN’S NEW ROLE as the unofficial standard-bearer of a new movement in U.S. science “is not who I had planned on being,” she said recently. She says her evolution has been “squarely tied” to her involvement in Vanderbilt’s investigation of a sexual harassment case, and what she believes were its consequences for her.

In the fall of 2014, McLaughlin submitted her tenure package, and the following year her department and VUMC’s Appointments and Promotions Committee recommended her for tenure, according to a later university report.



But the university halted her tenure process in December 2015, in the wake of allegations that arose during the investigation of a colleague. In early July 2014, former graduate student Erin Watt sued her former Ph.D. supervisor, neuroscientist Aurelio Galli, who was then at the Vanderbilt School of Medicine. Watt alleged in the lawsuit that Galli had sexually harassed and belittled her, leading her to quit the Ph.D. program.

In late July of that year, McLaughlin, her then-husband (a Vanderbilt neuroscientist at the time, who collaborated with Galli), and a visiting McLaughlin friend and collaborator, Dana Miller of the University of Washington in Seattle, were invited to dinner at Galli’s home. Miller and McLaughlin later recalled that while preparing dinner, Galli threatened to “destroy” Watt. Miller recalled him calling Watt “a crazy bitch” and vowing to “spend every last dime” to ruin her. The women say Galli showed them a handgun and noted that he had a permit to carry it. Miller, a lesbian, also told investigators that Galli made inappropriate comments about her sexuality.

Galli, now at the University of Alabama in Birmingham, declined to comment on the dinner party. But he told *Science*: “I have never done anything to any student or any faculty in terms of harassment or retaliation.” He provided an email that McLaughlin sent him the day after the party: “Dinner was fantastic. ... Thank you,” she wrote with a smiley face.

In December 2014, a judge dismissed Watt’s lawsuit against Galli and he was immediately promoted. (Watt settled with

Vanderbilt University, which she had also sued.) Miller says she was alarmed by Galli’s promotion, and in January 2015 reported the alleged events of the July 2014 dinner to a Vanderbilt administrator. McLaughlin testified in the ensuing investigation, backing up Miller’s account. In August 2015, investigators determined that the evidence they had obtained could not support a finding of harassment, according to a letter to Miller from Vanderbilt’s Equal Opportunity, Affirmative Action, and Disability Services Department (EAD).

Meanwhile, Galli alleged to EAD investigators that McLaughlin was sending derogatory tweets about unnamed colleagues, including him, from anonymous, multiuser Twitter accounts. McLaughlin admitted writing a tweet that predated the dinner party, complaining, “Galli has 3 R01 [grants] and whines about being broke.” A tweet apparently directed at another person—described as the “hateful” principal investigator across the hall—said, “I may stab her today.” McLaughlin told *Science* that she does not recall authoring such a tweet. (Four current or former Vanderbilt faculty members reported to have clashed with McLaughlin and contacted by *Science* declined to comment or did not return interview requests.)

EAD questioned McLaughlin about the tweets, and an associate medical school dean launched a disciplinary investigation of her, according to university documents. During that probe, which stretched from December 2015 to April 2017, McLaughlin’s tenure process was frozen. She stopped

"We have faculty members who have grabbed and groped and raped and assaulted and retaliated and diminished women. And they can be teaching your classes. It's not OK."

BethAnn McLaughlin, Vanderbilt University Medical Center

writing grant applications and taking on graduate students, she says; she felt her future was too uncertain. Her lab shrank. "It had a real impact on my research," she says of the investigation. "My career has been significantly diminished in what should have been the most productive and fun and creative time."

An outside law firm hired by Vanderbilt concluded that McLaughlin "more likely than not" had written the "I may stab her" tweet, according to university documents. But the faculty disciplinary committee split two to one in her favor, and administrators did not discipline McLaughlin. They restarted her tenure process, and VUMC's Executive Committee of the Executive Faculty approved her tenure in summer 2017.

But Vanderbilt School of Medicine Dean Jeffrey Balser asked the committee to reconsider. According to university documents, he circulated the faculty disciplinary report to the committee, which then met in person. This time, it voted unanimously to deny tenure. Seven of nine members later said they did not consider the disciplinary report in their decision, according to university documents.

"Faculty familiar with the bar at Vanderbilt in her department and at the institutional level determined she was up to snuff and voted her up for tenure," says Valina Dawson, a neuroscientist at the Johns Hopkins School of Medicine who knows McLaughlin because both are reviewing editors at *The Journal of Neuroscience*. "Then the dean decided to intervene. ... You get a bad feeling in your gut about the whole process."

In November 2017, McLaughlin filed a grievance, which is still being considered by a faculty committee. (Jeremy Berg, editor-in-chief of *Science* in Washington, D.C., wrote a letter last fall in support of McLaughlin, saying her activism had "accelerated" AAAS's decision to develop a process for potentially stripping fellowships from harassers.) A McLaughlin lawyer, Ann Olivarius of McAllister Olivarius in Saratoga Springs, says McLaughlin's case "is a perfect example of the tactics that universities so often use to sweep complaints under the rug."

Spokespeople for both Vanderbilt and VUMC said they could not comment on tenure decisions or grievances, and VUMC declined to make Balser, now its president and CEO, available to comment. Their emailed statement added that, "VUMC is committed to establishing and maintaining an open, inclusive, and equitable environment ... [and] fostering an open and civil exchange of diverse ideas and viewpoints."

Now, McLaughlin is waiting to learn what the grievance committee recommends. Whether the chancellor and Board of Trust concur will determine her future at VUMC.

AS HER TENURE BATTLE continued, McLaughlin grew more outspoken as *Fighty Squirrel*. Then, in May 2018, she read an account of alleged sexual harassment spanning 40 years by leading cancer scientist Inder Verma (*Science*, 4 May 2018, p. 480). Verma, who denied the allegations, resigned from the Salk Institute for Biological Studies in San Diego, California, in June 2018. With a few clicks, McLaughlin realized that Verma remained an NAS member in good standing. That, she says, was when she decided to go public.

McLaughlin began to campaign for change from her readily identifiable Twitter account, @McLNeuro. "You cannot ACTIVELY CONTRIBUTE TO FURTHERING SCIENCE IN AMERICA if you have impeded the progress of women by harassing, assaulting and retaliating against them. Period. Kick ... Verma out of National Academy of Sciences," she tweeted days after the story ran online. The next day, she launched a petition that has been signed by 5700 people, urging NAS to eject members who have been sanctioned for sexual harassment.

The next month, NAS published a landmark report documenting high rates of sexual harassment in STEM and noting that many funding agencies haven't taken "meaningful action" (*Science*, 15 June 2018, p. 1159). In August 2018, McLaughlin launched another petition, which has since drawn 2400 signatures, urging NIH's Collins to deny awards and other privileges to proven harassers. "TimesUp

Francis," it says. When she met with Collins last week, McLaughlin says, he apologized for not responding more quickly to sexual misconduct by grantees. "Apologies and involving #MeTooSTEM survivors are important first steps," she tweeted alongside a photo of herself with Collins. "Lots to do."

Separately, McLaughlin is keeping the pressure on NAS. In December 2018, she tweeted to NAS President Marcia McNutt, "Why don't you quit @Marcia4Science and I'll take over and show some decency?"

As her profile rose, McLaughlin found herself receiving thousands of requests for help; since April 2018, she says she has counseled—often by telephone in the wee hours—more than 200 sexual harassment survivors and witnesses.

One, Debra DeLoach, is a Ph.D. student at the University of Bath in the United Kingdom whom McLaughlin contacted in December 2018, after DeLoach tweeted that she had attempted suicide after being sexually harassed. "What she gave me was a sense of empowerment: the ability to narrate and write my own story. It helped me to reclaim my sense of agency," DeLoach says.

McLaughlin makes no apologies for her profane style. "We have faculty members who have grabbed and groped and raped and assaulted and retaliated and diminished women. And they can be teaching your classes. It's not OK," she says.

But her in-your-face delivery unnerves some establishment supporters. The Society for Neuroscience (SfN) gave her an award for "significantly promoting" women at its annual meeting in San Diego in November 2018 and invited her to speak to a virtual conference last month. But after McLaughlin prerecorded her comments, she received a letter from an SfN lawyer disinviting her. "Some of the content [in your presentation] may be defamatory and ... could expose the society to potential legal liability," it said.

McLaughlin promptly launched a stream of Twitter fire. "Too bad @SfNtweets kicked me off their Gender and Diversity panel this week," she tweeted. "Rebels and truth sayers need not speak." (SfN declined to comment.)

McLaughlin says she's booked with speaking engagements through next fall, and she's dealing with a legal case: In October 2018, Galli sued her for defamation.

But she keeps making people laugh. On the last day of the Banbury meeting, participants arrived in the conference room to find that the wall photos of famous male scientists had been covered with paper photos of female scientists, famous and not. Among them: Nobel laureates Marie Curie and Carol Greider, and BethAnn McLaughlin. ■



POLICY FORUM

CONSERVATION

Long delays in banning trade in threatened species

Scientific knowledge should be applied with more urgency

By Eyal G. Frank¹ and David S. Wilcove²

The harvesting of wild animals and plants for international trade affects thousands of species, and compounds ongoing extinction threats such as habitat loss and climate change (1–4). The loss of overexploited species can result in cascading effects that reduce overall ecosystem functioning (4, 5). The primary international framework for preventing the loss of species due to international wildlife

trade is the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Given that CITES aims to be as scientifically based as possible (6), we analyzed how quickly species that are identified by the International Union for Conservation of Nature (IUCN) Red List as being threatened from trade are subsequently protected under CITES. The Red List represents an authoritative body of scientific knowledge regarding extinction risks. We find that in nearly two-thirds of the cases, the CITES pro-

cess of regulating trade in threatened species lags considerably behind the IUCN identification of species in need of protection from trade. Such delay in the application of scientific knowledge to policy formulation could result in species extinctions. With signatories to CITES set to gather in May to determine which species merit protection, we suggest opportunities to improve this process.

IMPORTANT TOOLS

The CITES treaty, which has been ratified by 183 party members, was formalized in 1973 and entered into force in 1975 in order to coordinate and regulate international trade in wildlife products. The strongest tool CITES has is to list a species in Appendix I, which restricts trade in that species to “exceptional circumstances” only (7). This, in effect, places a trade ban on specimens or their body parts that are caught in the wild for commercial purposes, although it still allows trade for

International wildlife trade can develop quickly, threatening species with extinction, such as this helmeted hornbill, in just a few years.

personal or scientific reasons, such as the shipping of pets and trophies or the moving of live specimens for captive propagation.

CITES can also list species in Appendix II, which requires monitoring of trade in those species. Trade in species listed in Appendix II requires an export permit after a determination that the level of trade is not detrimental to the survival of the species and that the specimens were obtained in a legal manner (under domestic laws). Signatories to the treaty meet every 2 to 3 years at a Conference of Parties (CoP) where they vote on listing decisions. Listing in Appendix I or II requires approval by a two-thirds majority of party members.

The overall effectiveness of CITES at protecting species from international trade remains an open empirical question. Placing restrictions on trade can potentially increase demand if doing so signals that the species might become extinct in the near future. Also, if trade shifts from legal to illegal markets, it becomes harder to monitor and enforce a trade ban. Nonetheless, CITES is the only global agreement of its kind and, we would argue, an important tool in stemming extinctions due to international trade.

Estimating the true degree of threat facing wildlife populations is challenging. The IUCN is widely considered to be the global authority on the extinction risk of different species, which it assesses using quantitative criteria and compiles into the Red List. The IUCN compiles data on factors that imperil species, such as population declines, habitat loss, and direct harvesting. These assessments follow a systematic process that aims to make them comparable through time and across taxonomic groups (8, 9). Species classified as Vulnerable, Endangered, or Critically Endangered (hereafter “threatened”) are considered to be at risk of extinction; a species identified as having “intentional use” as a threat is one that is being directly targeted by collectors, hunters, or trappers.

With growth in international commerce and wildlife markets, coupled with unsustainable levels of legal and/or illegal trade, species can become endangered quickly (1, 2). For example, the helmeted hornbill (*Rhinoplax vigil*) was listed as only Near-Threatened in the Red List in 2012, but a sudden increase in demand around 2011 resulted in it being upgraded to Critically

Endangered in 2015 (10). The Tapah Islands race of the white-rumped shama (*Copsychus malabaricus opisthochrus*) went from being common to nearly extinct in the wild after only 5 to 7 years of intensive trapping for the pet trade (10). An estimated one million pangolins (Manidae) were trafficked from 2000 to 2013 (11). Although all pangolin species had been added to Appendix II by 2000, with trade quotas for some species set to zero, seven of the eight species were added to Appendix I only in 2017 in the face of rapidly escalating international trade.

IUCN ASSESSMENT AND CITES LISTING

We collected data on how species targeted by the international wildlife trade were classified by the IUCN and treated by CITES (12, 13) (see supplementary materials for details). We started with species that the IUCN has assessed as Vulnerable, Endangered, or Critically Endangered and for which the IUCN has listed direct harvesting (intentional use) as a threatening factor. We then drew information from IUCN assessments, academic articles, and reports to determine how many of these species were involved in international trade (eliminating those for which direct harvesting appeared to be for domestic use only).

This resulted in 958 threatened species for which we can link international trade as a factor in their endangerment. Because CITES held its two most recent CoPs in 2013 and 2016, we restricted our data to IUCN status assessments from 1994 to 2013, to ensure that assessments were based on the IUCN’s more rigorous criteria implemented in 1994 (14) and to allow CITES a minimum of 3 years to respond to the IUCN assessments. Information spanning from 1975 to 2018 is presented in fig. S1 and table S3.

Of the 958 threatened, internationally traded species that warranted CITES protection under Appendix I or II, 28.18% were not listed in either appendix. This is a striking, heretofore unrecorded gap in protection from international trade. There were, however, notable differences in protection relative to the severity of harvest pressure. The Red List assesses the severity of harvesting by assigning species an intentional use impact score of 0 (lowest rate of harvest but harvest definitely occurring) to 9 (highest). For each impact score, we determined the percentage of Red List threatened species that actually received protection under CITES Appendix I or II (see the figure, top).

For threatened species for which direct harvesting is an extremely severe threat (impact score 9) and which can be linked with international trade, 75% of species were listed in Appendix I, 20.83% were listed in Appendix II, and 4.17% were not listed in either appendix. All of the Red List’s En-

dangered and Critically Endangered species that are traded internationally and have an intentional use impact factor of 9 are listed in CITES Appendix I or II; only one Vulnerable species with an intentional use impact score of 9 remains unlisted. Thus, trade in the most highly exploited and threatened species has indeed been banned or restricted via CITES. However, for species with impact scores of 8 and below, there are many Endangered and even Critically Endangered species that, to date, have received no protection under CITES.

Beyond examining whether species receive protection under CITES, there is the issue of how long it takes them to receive that protection. We focus on three categories: (i) species assessed by the IUCN as threatened at least partly by international trade and subsequently protected by CITES, (ii) species assessed as threatened at least partly by international trade and not protected by CITES (as of 2018), and (iii) species that CITES protected ahead of any Red List determination that they were threatened at least partly by international trade.

We also summarize the gap (in years) between when each species was assessed by the IUCN as threatened and when it was protected under CITES (see the figure, bottom). Out of 958 species that the Red List classifies as threatened due to intentional use and which are traded internationally, 271 (28.18%) lack CITES protection, 334 (34.86%) received CITES protection after they were assessed by the Red List, and 353 species (36.84%) were protected under CITES before they were assessed by the IUCN as being threatened by international trade.

For this last group of 353 species, it is possible that the parties to CITES had access to information indicating threats posed by trade before such information was available to the IUCN. However, it is also the case that many of the species listed by CITES ahead of the IUCN were the result of higher taxonomic groups (for example, entire genera or families) being added en masse to Appendix I or II. This is sometimes done to ensure that threatened species cannot be easily mislabeled as similar-looking, nonthreatened species and therefore slipped into trade.

Moreover, the process of evaluating species for the Red List is subject to constraints on staffing, funding, or the gathering of scientific information. As a result, certain taxonomic groups have been evaluated by the Red List only relatively recently (15). Thus, the large outlier of 127 species added to CITES 18 years before they were assessed by the IUCN (see the figure, bottom) is driven by one group of species: CITES listed 118 corals (class Anthozoa) in 1990, whereas the IUCN did not assess corals until 2008. Mammals were mostly

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assessed and added to the Red List after 1996 and in 2008, and amphibians were mostly assessed and added in 2004.

We also identified 96 and 747 species listed on CITES Appendices I and II, respectively, that are also considered threatened by the IUCN yet are not classified by the IUCN as having intentional use as a threat factor. This may reflect an incomplete cataloging of threats to these species by the IUCN.

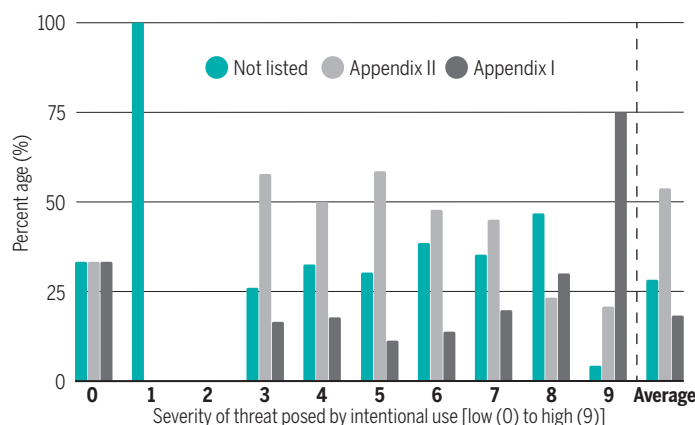
On average, when the IUCN preempts CITES, CITES lists species in Appendix I or II 10.3 years after the IUCN assesses them as being threatened by international trade. When CITES preempts the IUCN, species are assessed as threatened by the IUCN 19.8 years, on average, after they were protected under CITES. For species that are considered by the Red List to be threatened by international trade but have yet to be protected under CITES, we cannot know how long they will have to wait to receive protection under Appendix I or II. For those species, we calculate their lag time as the time since their listing as threatened on the Red List until the end of 2018, which is, on average, 12.4 years. We obtain similar results when focusing on internationally traded Endangered and Critically Endangered species with intentional use impact scores of 6 and above (fig. S2).

We consider the situation in which CITES protection is delayed relative to the Red List finding to be a more severe problem than the reverse situation, when CITES acts ahead of the Red List. The IUCN Red List is purely informational and provides no legal protection to imperiled species, whereas CITES does. Although a species' Red List status by itself does not convey any legal protection, it can play an important role in the allocation of conservation resources and in drawing attention to the species' plight.

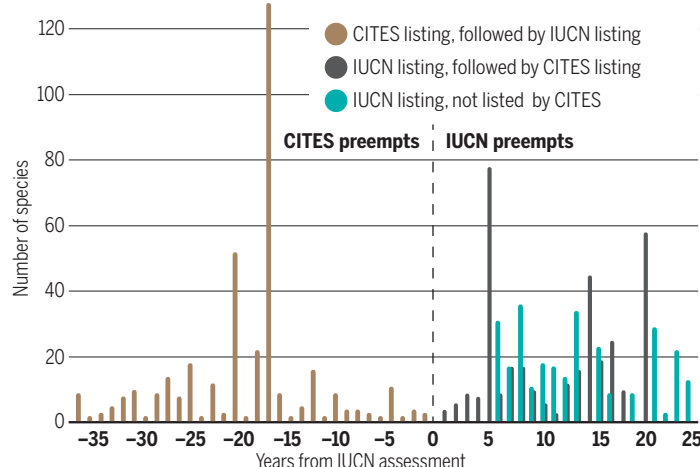
It is important to note that listing under CITES is not sufficient by itself for conservation. CITES coordinates efforts between countries, facilitates the flow of information, and aggregates reports about the levels of trade, but the party members must implement steps through their respective enforcement institutions for there to be real protections.

Overlap and lags between CITES and IUCN listings

Percentage of species under escalating degrees of threat from trade (intentional use impact score) that are listed in CITES Appendix I or II, or are not listed. See figs. S3 and S4 for additional details.



The number of species that CITES protected before (brown) or after (gray) an IUCN assessment as threatened by trade as well as the number of species that IUCN assessed as threatened by trade but which CITES has not yet protected (teal).



RECOMMENDATIONS

To increase the efficacy by which overexploited species receive protection, we offer three recommendations. First, independently from CITES, all countries can use the Red List as a source of information and take measures to protect threatened species found within their borders, including protection from trade. Also, in addition to coordinating trade restrictions, CITES identifies data collection needs and acts as a repository for data; countries can contribute to this process.

Second, hundreds of species that the IUCN classifies as Critically Endangered, Endangered, or Vulnerable due to international trade currently lack CITES protection. To begin to clear this backlog, any signatory nation (party) to CITES can propose that these species be added to Appendix I or II at the next CoP [per Article IV(1) (a)]. Failing that, the CITES Secretariat can at least alert the signatories to the plight

of such species under Article XII(2)(e). The ultimate goal should be to create a near-automatic pathway by which unprotected species identified by the IUCN as threatened by international trade receive a prompt vote for inclusion in CITES Appendix I or II.

Third, species that are listed in CITES Appendix I or II but are not classified by the IUCN as threatened should be reassessed by the IUCN as soon as practicable, in case CITES has identified a growing trade threat ahead of the IUCN. Taken together, these steps will improve the degree to which conservation science informs conservation policy and may help to avert the extinction of species due to escalating international trade in wildlife. ■

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SUPPLEMENTARY MATERIALS

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Revealing a microbial carcinogen

An *E. coli*-derived colibactin-DNA adduct is detected in intestinal tissues

By Rachel M. Bleich¹ and
Janelle C. Arthur^{1,2,3}

The microbiota in the human gastrointestinal system is predicted to produce hundreds of unique small molecules and secondary metabolites that may influence host health and disease (1). Many such molecules are produced by sophisticated multienzymatic assembly lines that are encoded by bacterial biosynthetic gene clusters. One class of molecules, colibactins, are produced from the gene cluster called the polyketide synthase (*pks*) island. The *pks* island occurs in certain strains of *Escherichia coli* and is prevalent in the microbiota of colorectal cancer (CRC) patients (2–5). However, despite more than a decade of research into the potential carcinogenic role of colibactin, little is known about its structure or mechanism of action. On page 709 of this issue, Wilson *et al.* (6) show that colibactin alkylates DNA in cultured cells and in vivo, forming covalent modifications known as DNA adducts. These colibactin-DNA adducts are chemical evidence of DNA damage and represent a detectable signature of exposure to colibactin. Misrepaired DNA adducts may generate mutations that contribute to colorectal tumorigenesis.

Colibactin was first described as an unknown product of a 54-kilobase genomic island that encodes a hybrid nonribosomal peptide synthetase–polyketide synthase gene cluster, the *pks* island, in some commensal and extraintestinal pathogenic *E. coli* strains (2). Exposure to *pks*⁺ *E. coli* induces DNA double-strand breaks and an increased gene mutation frequency in mammalian cells in culture (3). This raised speculation that products of *pks* were microbial-derived genotoxins that could promote cancer. The tumorigenic potential of *pks* products was demonstrated in a study showing that *pks*⁺ *E. coli* was abundant in colon tissue from CRC patients and promoted CRC in mouse models (4). This tumorigenic effect was later demonstrated in several other mouse models of CRC (5, 7, 8).

Because of its instability, the structure of colibactin has been elusive (2). Most previous work to determine the structure of colibactin has focused on identifying stable precursors using mutant strains of *E. coli* missing the colibactin-producing peptidase ClbP, which activates colibactin precursors by removing an *N*-myristoyl-D-asparagine “prodrug group” (9). However, the precursors do not necessarily represent a final colibactin structure. Previous research suggested that colibactin alkylates DNA and forms a DNA adduct via a cyclopropane functional group, called the “warhead,” which is structurally similar to other natural products that alkylate DNA (9, 10). The importance of the cyclopropane ring was confirmed by identification of the colibactin resistance protein ClbS, which inactivates the cyclopropane ring to provide self-protection against DNA damage in the *pks*⁺ bacteria (11). Recently, colibactin-DNA adducts with similar structures were detected in vitro using purified DNA and colibactin-producing bacteria (10). However, there was no direct evidence or structural characterization of these colibactin-DNA adducts in a biological setting.

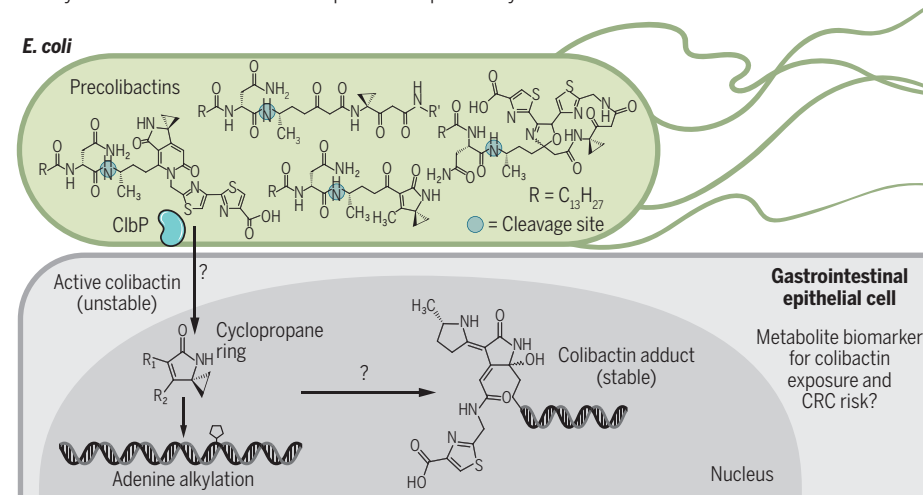
Wilson *et al.* used an untargeted mass spectrometry DNA adductomics approach

to structurally and mechanistically define a DNA alkylation end product of colibactin exposure. They identified two stereoisomeric colibactin adducts to the DNA nucleotide adenine in cultured mammalian cells and in colonic epithelial cells of formerly germ-free (sterile) mice colonized with a single *pks*⁺ *E. coli* strain, providing direct evidence that these DNA adducts occur in vivo. As the authors note, the structure they uncovered does not necessarily represent the immediate colibactin-DNA adduct but is likely a degradation product of a larger colibactin adduct. This study provides important information about the structure and mechanism of action of colibactin. Furthermore, it describes a mass spectrometry method that could be used to identify other intractable compounds.

The adenine-colibactin adducts elucidated by Wilson *et al.* provide insight into how the cyclopropane functional group could react to alkylate DNA so effectively. These structures support a reaction mechanism whereby the cyclopropane ring is conjugated to an α,β -unsaturated imine formed from an intramolecular cyclodehydration that occurs once ClbP deacetylates the prodrug group. The presence of this proposed imine increases the reactivity of the cyclopropane ring to alkylate DNA

Model of colibactin-induced CRC

Precolibactins are synthesized from the *pks* island in *E. coli* before being activated by ClbP. When *E. coli* has direct contact with a mammalian cell, data suggest that the unstable, active colibactin reaches the nucleus where it alkylates DNA. A stable colibactin-DNA adduct was identified by Wilson *et al.*, revealing the structural identity of a biomarker for colibactin exposure and potentially for CRC risk.



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(12) (see the figure). This alkylation generates DNA adducts that could lead to mutations in oncogenes or tumor suppressors that drive CRC tumorigenesis.

The identity of colibactin has been a long-standing question in the field of microbiota-influenced CRC. An important question to be resolved by further studies is how to distinguish the precise type of DNA damage responsible for the carcinogenic effects of colibactin. For example, what are the kinetics and relative levels of monoadducts versus interstrand DNA cross-links that can also result from alkylation and have been shown to occur after exposure to *pks*⁺ *E. coli* (6, 13)? Many other questions remain. For example, many bacterial biosynthetic gene clusters produce several bioactive molecules; is more than one colibactin variant produced from the *pks* island? Also, are there other roles for colibactin in mediating the interaction between the bacteria and human host? Undoubtedly, *E. coli* did not acquire *pks* to destroy its ecosystem by inducing DNA damage that may lead to cancer; instead, it is likely that *pks* imparts an important microbiological function, such as colonization and persistence in the gut (14).

From a clinical perspective, is there a way to predict which resident *E. coli* strains will colonize the gut mucosa and permit colibactin delivery? Colibactin requires direct cell-to-cell contact to exert its genotoxicity (2); thus, how does colibactin get from the bacteria into the nucleus of gastrointestinal epithelial cells, where it can cause DNA damage? Finally, how can we further apply our knowledge to improving clinical outcomes and treatment? This work has revealed a potential metabolite biomarker for colibactin exposure: adenine-colibactin adducts. However, it remains unknown whether adenine-colibactin adducts can distinguish precancerous tissue from healthy epithelium. We also do not yet know whether misrepaired adenine-colibactin adducts lead to gene mutations associated with known CRC subtypes and/or response to therapy. Future studies and the structural insight provided by Wilson *et al.* are expected to provide the next step toward applying microbiota signatures to improve prognosis and treatment for CRC. ■

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STRUCTURAL BIOLOGY

Pathology-linked protease caught in action

Structural snapshots of γ -secretase yield insight for drug development

By Stefan F. Lichtenthaler
and Gökhan Güner

The intramembrane protease γ -secretase has fundamental functions in animals, including signal transduction during embryogenesis and tissue homeostasis in adulthood. γ -Secretase cleaves its numerous substrates within their single transmembrane domains (TMDs), largely independently of their amino acid sequence. Abnormal cleavage of the substrates Notch and amyloid precursor protein (APP) is linked to leukemia and Alzheimer's disease (AD), respectively, making γ -secretase an important drug target for both diseases (1). Yet, chronic use of γ -secretase inhibitors (GSIs), such as in patients with AD, led to severe side effects, resulting from cleavage inhibition not only of the disease-relevant substrate APP but likely also of other substrates. Thus, there is a clear need to develop substrate-selective GSIs, but this requires a detailed understanding of how γ -secretase recognizes, binds, and cleaves its substrates. On page 708 of this issue, Zhou *et al.* (2) and another study by Yang *et al.* (3) provide a major step in this direction. Zhou *et al.* reveal the cryo-electron microscopy (cryo-EM) structure of human γ -secretase with its bound substrate, a fragment of APP. Yang *et al.* report a structure of γ -secretase, but bound with Notch. Together, the two studies demonstrate that binding of different substrates occurs in a similar manner and that both γ -secretase and substrate undergo specific structural rearrangements for substrate positioning in the active site. This has major implications for understanding the mechanism of γ -secretase and its function in signal transduction and AD, and for future development of substrate-specific GSIs with fewer side effects.

The aspartyl protease γ -secretase consists of four integral membrane protein subunits (4). The subunit presenilin (PS) contains the active site aspartyl residues (5) and exists in two variants, PS1 and PS2. Another sub-

unit, nicastrin, has a tightly folded extracellular domain, which forms a lid on top of the membrane-bound γ -secretase complex. PEN-2 and APH-1A or APH-1B are additional subunits required for correct assembly, maturation, and trafficking of γ -secretase to the plasma membrane and endosomes.

Detailed biochemical analysis revealed that substrate cleavage by γ -secretase requires the substrate to move through an amazingly complex multistep process (see the figure). A substrate needs to have a short extracellular domain, either naturally (6) or as a result of an initial proteolytic cleavage (7), which is independent of γ -secretase and removes a large part of the extracellular domain, as for Notch and APP. This helps the substrate to fit below the lid imposed by nicastrin (8) and is considered a regulatory step to ensure that membrane proteins are only cleaved by γ -secretase when needed (7). Next, the truncated substrate likely binds to exosites outside of the active site of γ -secretase (9), followed by transfer to the active site, where cleavage occurs at the so-called ϵ site, a peptide bond close to the carboxyl-terminal end of the TMD in the substrate. Subsequently, the TMD is further truncated in a stepwise fashion up to the middle of the TMD (10), which is referred to as the final γ cut. If a membrane protein fails any of the requirements up to the ϵ cleavage, it will not be a substrate for γ -secretase.

Previously, a cryo-EM structure of γ -secretase was reported (11), but without the substrate, which was difficult to co-isolate. To achieve this, Zhou *et al.* and Yang *et al.* used two elegant tricks. First, they introduced single cysteine mutations into PS1 and either of the two substrates, derived from APP or Notch. The cysteines did not affect the activity of the protease or cleavage of the mutated substrate but allowed a stable cross-link between substrate and protease, essential for copurification. Second, one of the two catalytic aspartate residues in the active site was mutated to an alanine, which is known to abolish γ -secretase activity (5) and prevented undesired substrate cleavage during protein purification. Although both mutations are a caveat, the structures are in line with previous predictions based on bio-

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chemical experiments (9) and thus present an excellent snapshot of how a substrate can be bound in the active site just before cleavage occurs. It is possible, though, that other substrates or native substrates without the forced cysteine cross-link show somewhat different structures.

A comparison of the substrate-bound structures to the known structure of γ -secretase without substrate and to the structure of the substrates without protease yields ex-

TMD1 of PS. Next, the amino terminus of the substrate TMD seems to tilt outward, which brings the scissile peptide bond at the carboxyl terminus, that is, the ε -cleavage site, in close proximity to the two catalytic aspartate residues in PS. This is accompanied by the arguably most remarkable structural change seen in both structures, namely the partial unfolding of the substrate TMD helix around the ε -cleavage site and its formation of a β -pleated sheet. This requires substan-

PS did not undergo major structural rearrangements upon substrate binding, suggesting that this is the reason why AD-causing inherited mutations have not been found in those subunits.

Another important implication is rational drug development of GSIs that preferentially block γ cleavage of APP over the cleavage of other substrates. This could now become possible and may revive the use of γ -secretase as an AD drug target. Similarly, Notch-specific

GSIs may reduce side effects in cancer treatment. Yet, the structures with Notch and APP are more similar than different, making rational drug development a longer-term goal. Potentially, the exosites, where Notch and APP initially bind to γ -secretase (9), are more different from each other and thus may offer additional opportunities for developing substrate-specific GSIs.

A future challenge is to understand how substrates with substantially longer amino termini than the investigated APP and Notch fragments—such as B cell maturation antigen (6), the AD-relevant C99 fragment of APP, or artificial Notch cleavage constructs with long ectodomains (8)—bind to γ -secretase and pass below the loop in PS. Another major task is to solve the

structure of additional substrate-bound intramembrane proteases, such as the rhomboids [which are distant PS homologs and members of the signal peptide peptidase-like (SPPL) family] and the site-2 proteases (S2Ps). Together, these structures will uncover the mechanistic secrets of how targeted proteolysis within the lipid bilayer controls cell signaling in health and disease. ■

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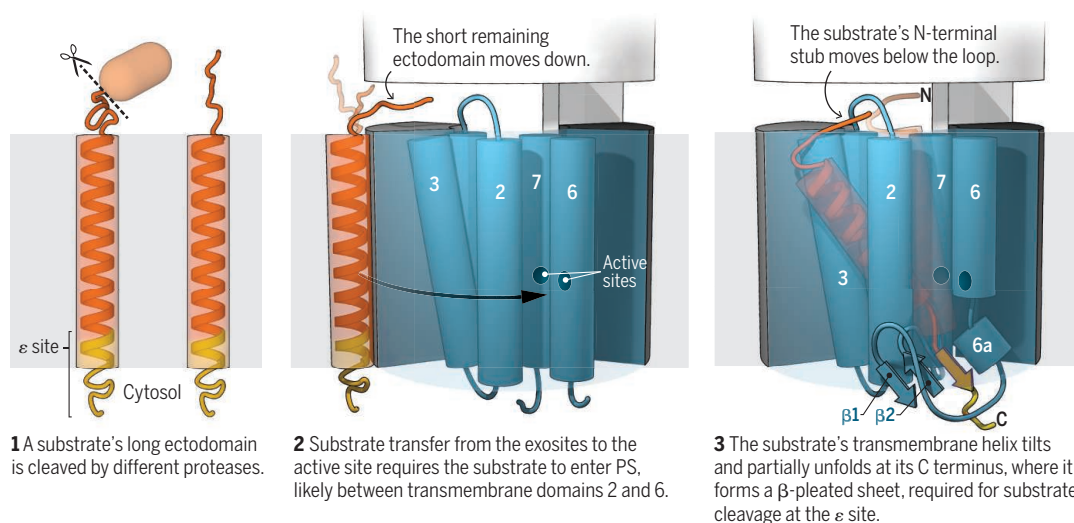
10.1126/science.aaw5547

Substrate binding by γ -secretase is a multistep process

The four γ -secretase subunits PS, PEN-2, APH-1, and nicastrin form a horseshoe-like structure in the membrane, with the nicastrin ectodomain forming a lid on top of the horseshoe structure. Insights from Zhou *et al.* and Yang *et al.* clarify the mechanism of substrate binding by γ -secretase.



● PS ● APH-1 ● PEN-2 ● Nicastrin ● Substrate



citing and partly unexpected insights into the molecular steps that are likely to have happened before the substrate arrived in the active site. Imagine nine transmembrane helices of PS that are linked by loops and form a well-ordered, compact structure within the membrane. Suddenly, they need to make space to integrate the substrate TMD, which requires substantial structural rearrangements in both γ -secretase and its substrate. This is in line with the induced-fit model of enzymology and may be one of the reasons why γ -secretase has slow enzymatic kinetics (12). Specifically, to fit below the nicastrin lid, the substrate ducks its head, which corresponds to a turn of the short amino-terminal substrate extracellular domain toward the membrane (see the figure). Then TMD2 and TMD6 of PS are likely to open up because they are an obvious lateral entry point of the substrate TMD into γ -secretase, although other entry points also appear to be possible (9, 13). Subsequently the still “ducked head” may allow the substrate to pass below the loop connecting TMD2 with

tial helix flexibility (14) and makes the scissile peptide bond accessible to cleavage. At present, the exact order of the above steps is unknown, but mutational studies suggest that formation of the β -pleated sheet is key to correctly position the substrate TMD in the active site (2, 3).

The new insights into substrate binding by γ -secretase have major implications beyond understanding the molecular operation mode of γ -secretase. Now, it is possible to better understand the molecular basis of dominantly inherited forms of AD, which are caused by point mutations at more than 100 different amino acid residues in PS1 or PS2 and partly around the γ -secretase cleavage site in APP (15). Mapping of the mutations onto the new structures suggests that many of them directly affect the interaction between APP and PS1, resulting in altered cleavage efficiency and higher levels of the pathogenic, AD-linked APP cleavage product A β 42 (2, 9). Final proof will come from future cryo-EM structures of mutated APP or PS1. Interestingly, γ -secretase subunits other than

NEUROSCIENCE

Using neuroscience to develop artificial intelligence

Combining deep learning with brain-like innate structures may guide network models toward human-like learning

By Shimon Ullman

When the mathematician Alan Turing posed the question “Can machines think?” in the first line of his seminal 1950 paper that ushered in the quest for artificial intelligence (AI) (1), the only known systems carrying out complex computations were biological nervous systems. It is not surprising, therefore, that scientists in the nascent field of AI turned to brain circuits as a source for guidance. One path that was taken since the early attempts to perform intelligent computation by brain-like circuits (2), and which led recently to remarkable successes, can be described as a highly reductionist approach to model cortical circuitry. In its basic current form, known as a “deep network” (or deep net) architecture, this brain-inspired model is built from successive layers of neuron-like elements, connected by adjustable weights, called “synapses” after their biological counterparts (3). The application of deep nets and related methods to AI systems has been transformative. They proved superior to previously known methods in central areas of AI research, including computer vision, speech recognition and production, and playing complex games. Practical applications are already in broad use, in areas such as computer vision and speech and text translation, and large-scale efforts are under way in many other areas. Here, I discuss how additional aspects of brain circuitry could supply cues for guiding network models toward broader aspects of cognition and general AI.

The key problem in deep nets is learning, which is the adjustment of the synapses to produce the desired outputs to their input patterns. The adjustment is performed automatically based on a set of training examples, which are provided by input patterns coupled with their desired outputs. The learning process then adjusts the weights to produce the desired outputs to the training input patterns. Successful learning will

cause the network to go beyond memorizing the training examples, and be able to generalize, and provide correct outputs to new input patterns, which were not encountered during the learning process.

Comparisons of deep network models with empirical physiological, functional magnetic resonance imaging, and behavioral data have shown some intriguing similarities between brains and the new models (4), as well as dissimilarities (5) (see the figure). In comparisons with the primate visual system, similarities between physiological and model responses were closer for the early compared with later parts of the neuronal responses, suggesting that the deep network models may capture better the early processing stages, compared with later, more cognitive stages.

In addition to deep nets, AI models recently incorporated another major aspect of brain-like computations: the use of reinforcement learning (RL), where reward signals in the brain are used to modify behavior. Brain mechanisms involved in this form of learning have been studied extensively (6), and computational models (7) have been used in areas of AI, in particular in robotics applications. RL is used in the context of an agent (a person, animal, or robot) behaving in the world, and receiving reward signals in return. The goal is to learn an optimal “policy,” which is a mapping from states to actions, so as to maximize an overall measure of the reward obtained over time. RL methods have been combined in recent AI algorithms with deep network methods, applied in particular to game playing, ranging from popular video games to highly complex games such as chess, Go, and shogi. Combining deep nets with RL produced stunning results in game playing, including convincing defeats of the world’s top Go players, or reaching a world-champion level in chess after ~4 hours of training, starting from just the rules of the game, and learning from games played internally against itself (8).

From the standpoint of using neuroscience to guide AI, this success is surprising, given the highly reduced form of the network models compared with cortical

circuitry. Some additional brain-inspired aspects, for example, normalization across neuronal groups, or the use of spatial attention, have been incorporated into deep network models, but in general, almost everything that we know about neurons—their structure, types, interconnectivity, and so on—was left out of deep-net models in their current form. It is currently unclear which aspects of the biological circuitry are computationally essential and could also be useful for network-based AI systems, but the differences in structure are prominent. For example, biological neurons are highly complex and diverse in terms of their morphology, physiology, and neurochemistry. The inputs to a typical excitatory pyramidal neuron are distributed over complex, highly branching basal and apical dendritic trees. Inhibitory cortical neurons come in a variety of different morphologies, which are likely to perform different functions. None of this heterogeneity and other complexities are included in typical deep-net models, which use instead a limited set of highly simplified homogeneous artificial neurons. In terms of connectivity between units in the network, cortical circuits in the brain are more complex than current deep network models and include rich lateral connectivity between neurons in the same layer, by both local and long-range connections, as well as top-down connections going from high to low levels of the hierarchy of cortical regions, and possibly organized in typical local “canonical circuits.”

The notable successes of deep network-based learning methods, primarily in problems related to real-world perceptual data such as vision and speech, have recently been followed by increasing efforts to confront problems that are more cognitive in nature. For example, in the domain of vision, network models were developed initially to deal with perceptual problems such as object classification and segmentation. Similar methods, with some extensions, are now being applied to higher-level problems such as image captioning, where the task is to produce a short verbal description of an image, or to the domain of visual question answering, where the task is to produce adequate answers to queries posed in natural language (that is, human communication) about the content of an image. Other, nonvisual tasks include judging humor, detecting sarcasm, or capturing aspects of intuitive physics or social understanding. Similar methods are also being developed for challenging real-world applications such as online translation, flexible personal assistants, medical diagnosis, advanced robotics, or automatic driving.

With these large research efforts, and the huge funds invested in future AI applica-

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tions, a major open question is the degree to which current approaches will be able to produce “real” and human-like understanding, or whether additional, perhaps radically different, directions will be needed to deal with broad aspects of cognition, and artificial general intelligence (AGI) (9, 10). The answers to this question are unknown, and the stakes are high, both scientifically and commercially.

If the success of current deep network models in producing human-like cognitive abilities proves to be limited, a natural place to look for guidance is again neuroscience. Can aspects of brain circuitry, overlooked in AI models so far, provide a key to AGI? Which aspects of the brain are likely to be particularly important? There are at

building upon specific preexisting network structures already encoded in the circuitry prior to learning. For example, different animal species, including insects, fish, and birds, can perform complex navigation tasks relying in part on an elaborate set of innate domain-specific mechanisms with sophisticated computational capabilities. In humans, infants start to develop complex perceptual and cognitive skills in the first months of life, with little or no explicit training. For example, they spontaneously recognize complex objects such as human hands, follow other peoples’ direction of gaze, and distinguish visually whether animated characters are helping or hindering others, and a variety of other tasks, which exhibit an incipient understanding of physi-

intermediate view are not developed concepts, but simpler “proto concepts,” which provide internal teaching signals and guide the learning system along a path that leads to the progressive acquisition and organization of complex concepts, with little or no explicit training. For example, it was shown how a particular pattern of image motion can provide a reliable internal teaching signal for hand recognition. The detection of hands, and their engagement in object manipulation, can in turn guide the learning system toward detecting direction of gaze, and detecting gaze targets is known to play a role in learning to infer people’s goals (14). Such innate structures could be implemented by an arrangement of local cortical regions with specified initial connectivity, supplying inputs and error signals to specific targets.

Useful preexisting structures could also be adopted in artificial network models to make their learning and understanding more human-like. The challenge of discovering useful preexisting structures can be approached by either understanding and mimicking related brain mechanisms, or by developing computational learning methods that start “from scratch” and discover structures that support an agent, human or artificial, that learns to understand its environment in an efficient and flexible manner. Some attempts have been made in this direction (15), but in general, the computational problem of “learning innate structures” is different from current learning procedures, and it is poorly understood. Combining the empirical and computational approaches to the problem is likely to benefit in the long run both neuroscience and AGI, and could eventually be a component of a theory of intelligent processing that will be applicable to both. ■

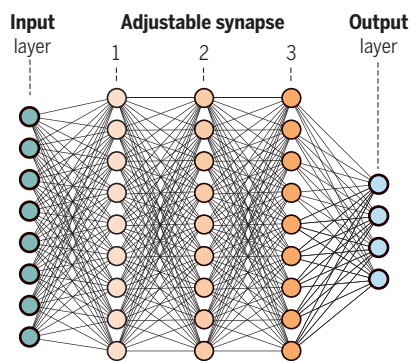
Brain circuitry and learning

A major open question is whether the highly simplified structures of current network models compared with cortical circuits are sufficient to capture the full range of human-like learning and cognition.



Complex neural network

Connectivity in cortical networks includes rich sets of connections, including local and long-range lateral connectivity, and top-down connections from high to low levels of the hierarchy.



Informed AI network

Biological innate connectivity patterns provide mechanisms that guide human cognitive learning. Discovering similar mechanisms, by machine learning or by mimicking the human brain, may prove crucial for future artificial systems with human-like cognitive abilities.

present no obvious answers, because our understanding of cortical circuitry is still limited, but I will briefly discuss a general aspect by which brains and deep network models appear to be fundamentally different and that is likely to have an important functional role in the quest for human-like AGI. The difference centers on the age-old question about the balance between empiricism and nativism in cognition, namely, the relative roles of innate cognitive structures and general learning mechanisms. Current AI modeling leans heavily toward the empiricist side, using relatively simple and uniform network structures, and relying primarily on extended learning, using large sets of training data. By contrast, biological systems often accomplish complex behavioral tasks with limited training,

cal and social interactions. A large body of developmental studies have suggested that this fast, unsupervised learning is possible because the human cognitive system is equipped, through evolution, with basic innate structures that facilitate the acquisition of meaningful concepts and cognitive skills (11, 12).

The superiority of human cognitive learning and understanding compared with existing deep network models may largely result from the much richer and complex innate structures incorporated in the human cognitive system. Recent modeling of visual learning in infancy (13) has shown a useful combination of learning and innate mechanisms, where meaningful complex concepts are neither innate nor learned on their own. The innate components in this

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CERAMICS

Hyperbolic 3D architectures with 2D ceramics

A hexagonal boron nitride aerogel has high resistance to thermal and mechanical shock

By **Manish Chhowalla¹** and **Deep Jariwala²**

Materials that operate in extreme environments, such as aerospace applications that require operation at high temperatures and in reactive atmospheres, must be ultralight, very mechanically strong, and thermally insulating. Achieving such disparate functionalities requires rational design not only of the material itself but also of hierarchical structures at multiple length

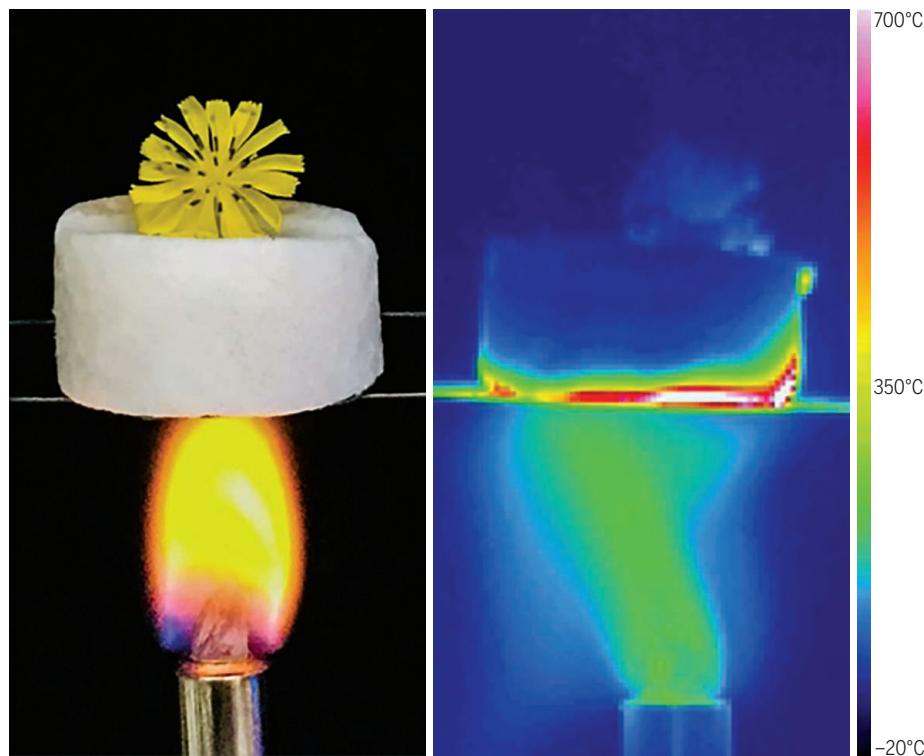
network of atomically thin sheets of hexagonal boron nitride (hBN). By careful mechanical design of the microstructure, the authors report that their aerogels exhibit extraordinary mechanical and thermal resistance properties far superior to those of current aerogels. Their discovery opens new pathways for the integration of rationally designed ultralightweight materials with the correct combination of mechanical and thermal properties for a variety of extreme environments.

gated for such applications, particularly in aerospace components that demand extreme material property requirements (3).

However, aerogels of typical ceramic materials such as silica, alumina, and silicon carbide are highly brittle and are fragile under stress, especially at high temperatures or under abrupt thermal shock (3). Conventional strategies for mitigating the brittleness of ceramic aerogels often lead to degradation of other properties, such as an increase in thermal conductivity. Unconventional strategies attempt to achieve a material that has both a negative Poisson's ratio (such that it would expand along the normal direction when stretched) and a negative thermal expansion coefficient (such that it would contract upon heating). These properties can be realized through internal hierarchical structuring (metamaterial architectures) that can in principle enhance the fracture toughness and mitigate the brittleness of ceramics. However, the synthesis of metamaterial aerogels with rationally designed hierarchical structures is challenging with bulk three-dimensional (3D) ceramics because of processing limitations.

Recent work on aerogels based on 2D graphene (a single atomic layer of graphite) provides the basic design principles for realizing ultralow-density, highly deformable, and thermally insulating aerogels (4, 5). Aerogels obtained from 2D materials feature a face-to-face stacking of the 2D nanosheets so that the cell walls of the aerogel consist of minimally thick material with exceptionally high mechanical strength. The 3D hierarchical structure obtained from 2D nanosheets also divides the aerogels into tiny cells so that convection of air between them is practically reduced, thereby enabling the realization of thermal conductivities below that of air.

Although graphene is not suitable for high-temperature applications in air, the fundamental knowledge obtained from its processability and design principles for achieving exceptional aerogel properties can be applied to other 2D materials to realize unexpected functionalities. Xu *et al.* have in fact rationally designed hyperbolic aerogels with hBN, a 2D ceramic. Their hBN aerogel comprises a defective crystal-line lattice of atomically thin planes interconnected to form a cellular network. To



Hyperbolic ceramics that keep their cool. An aerogel made from thin hexagonal boron nitride sheets by Xu *et al.* has exceptional mechanical strength and thermal insulation properties. This material (white block) can insulate a flower from the heat of an open flame for several minutes.

scales that can respond in the desired way to extreme environmental factors in real time. On page 723 of this issue, Xu *et al.* (1) report the synthesis of a multifunctional structure with hyperbolic surfaces (saddle shapes with negative curvature) in the form of an aerogel where the solid medium is a

Aerogels are mixtures or composites of air or free space and a ceramic, metal, particulate, powder, or carbon solid medium, where the proportion of air or free space is >99%. Thus, aerogels can be exceptionally lightweight with densities approaching 0.1 mg cm⁻³ (2). Ceramic aerogels possess many of the requisite properties for operation at high temperatures in corrosive environments, such as low density, excellent thermal insulation, and chemical stability. As a result, ceramic aerogels have been widely investi-

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synthesize the aerogel, they fabricated graphene aerogels using a previously reported technique (6) for use as sacrificial templates upon which they grew hBN layers. Because hBN is highly resistant to oxidation and its thermal stability in air is superior to that of graphene, the graphene template can be easily removed by oxidation to leave behind pure hBN aerogel.

For atomically thin walls, the aerogel density can be as low as 0.1 mg cm^{-3} , lower than most known solids. The aerogel also exhibits ultrahigh elastic deformation (up to 95%) and specific surface area ($>1080 \text{ m}^2 \text{ g}^{-1}$). Most important, it provides ultralow thermal conductivity (see the figure) concurrently with thermal shock resistance for several hundred cycles, making it highly attractive for extreme applications such as thermal shields of space vehicles. Xu *et al.* show that

“Xu *et al.* have in fact rationally designed hyperbolic aerogels with hBN, a 2D ceramic.”

the hyperbolic double-paned aerogel cell walls allow realization of a negative coefficient of thermal expansion and a negative Poisson's ratio. Both of these properties are opposite to conventional materials and are only achieved via careful engineering of the material and its structure (7, 8).

In addition to its extreme thermal shielding, high surface area, and refractory nature, the atomically thin-walled aerogel realized by Xu *et al.* serves as a starting point for a new class of 3D structures realized from 2D materials for applications requiring architectures with high surface-to-volume ratios, including catalysis and electrochemical energy storage. Further, the optical properties of these and aerogels made from other 2D semiconducting materials remain unexplored; if they can be engineered to have sufficiently low absorption, they become suitable structural material candidates for laser sails and directed-photon propulsion systems, such as those being proposed for interstellar probes (9). ■

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SURFACE CHEMISTRY

Moving through the crowd

Atoms or molecules can diffuse rapidly on surfaces covered by other adsorbants

By Olaf M. Magnussen

Chemical processes generally involve the transport of atoms and molecules, which on the nanoscale is dominated by diffusion. In interface chemistry, diffusion along surfaces and interfaces is an elementary and often determining step. For example, it plays a central role in crystal growth and dissolution, the deposition of thin films and nanostructures, and the self-assembly of two-dimensional organic layers. It also governs the transport of the reacting species on the surfaces of catalysts and thus the speed by which these species intermix or reach specific sites, where the catalytic reaction occurs. On page 715 of this issue, Henß *et al.* show that this transport can be rapid even in the presence of a layer of other adsorbants on the surface (1, 2).

Surface diffusion has been extensively studied for chemically bound atoms and small molecules that cover a small fraction of an otherwise clean solid surface (3). These adsorbates typically move on the surface by jumping between neighboring binding sites (see the figure, top left). During a jump, they must transiently occupy positions in which they are bound less strongly. This leads to an energy barrier, which the adsorbates overcome through thermal excitation. At a given temperature, the rate by which jumps between different positions on the surface lattice occur thus depends on the spatial variation of the binding energy; that is, it is exclusively determined by the interactions of the adsorbate with the atoms of the underlying solid.

This simple picture is, however, usually insufficient to fully describe surface diffusion in the real world. Even if only one species is present on the surface, the adsorbates may encounter other adsorbates during their random walk on the surface and interact with those. In the simplest case, the only effect of this is that positions already occupied by adsorbates are not available to others, a concept called “site blocking.” In reality, however, the adsorbates additionally attract or repulse each other, resulting in modified energy barriers for diffusion in the vicinity of

other adsorbates. If the surface fraction covered by adsorbates is high, these effects can lead to changes in the adsorbates mobility by orders of magnitude (4).

Similar effects may be expected for the motion of adsorbates on a surface covered by another atomic or molecular species, which Henß *et al.* address in their report. The diffusion of adsorbates on surfaces crowded by other coadsorbed species is the normal case for interface processes in ambient or high-pressure gas and liquid phases. However, despite its prevalence in technological systems and natural environments, it has been studied only sparsely and is not well understood. For this reason, the influence of coadsorbates on surface diffusion has mostly been either ignored or treated by simplified concepts such as site blocking.

In the past decade, the complexity of this situation has been revealed in some in-depth studies of adsorbate systems with well-defined composition and geometry (5, 6). These studies combine experimental data on surface diffusion, obtained from video sequences of atomic-resolution scanning tunneling microscopy images, with density functional theory calculations of the pathways by which the adsorbates move between neighboring sites. Hsieh *et al.* have investigated the diffusion of hydrogen on a chlorine-covered silicon surface and observed a direct exchange of the hydrogen atoms with neighboring chlorine atoms (5). They attributed the surprisingly low energy barrier for this exchange diffusion to the transient formation of a more weakly bound HCl adsorbate. Rahn *et al.* studied the diffusion of sulfur atoms on copper electrodes that were immersed in aqueous solution and covered by dense halide adlayers (6). They found an opposite dependence of the sulfur diffusion rates on the electrode voltage for sulfur coadsorbed with chloride and bromide, respectively, indicating different diffusion mechanisms for the two coadsorbates.

In both studies, the diffusing adsorbates and the surrounding coadsorbate were bound in an identical geometry to the surface and had the same distances to neighboring adsorbates (see the figure, top right). The diffusion processes can then be conveniently described via motions on one lattice that is common to both species. However, adsorbate and coadsorbate often bind differently to the surface and thus do not occupy iden-

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tical adsorption sites. How surface diffusion proceeds in this more complex case is the central topic of the report by Henß *et al.*

As an example, they chose the diffusion of oxygen atoms on a ruthenium surface covered by carbon monoxide, which is an important model system with relevance to the catalytic oxidation of CO. On the hexagonal surface lattice of the basal plane of Ru, oxygen atoms occupy a hollow site, situated between three neighboring Ru atoms, whereas CO is located on top of a Ru surface atom. Because of this mismatch, three symmetrical oxygen-binding sites exist within the cage formed by the surrounding CO (see the figure, bottom). Jumps between the three positions within the cage are not impeded by the CO coadsorbates. They thus occur much more frequently than jumps to the other neighboring binding sites on the Ru surface, which require relocation of a CO molecule. Nevertheless, the latter jumps also occur at a surprisingly high rate—nearly as high as on the CO-free Ru surface.

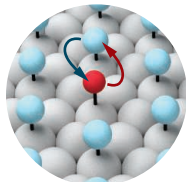
Diffusion on surfaces

Coadsorbates normally hinder diffusion of other adsorbate atoms or molecules. As Henß *et al.* show, density fluctuations in the adsorbate layer still can enable rapid surface diffusion.



Clean surface

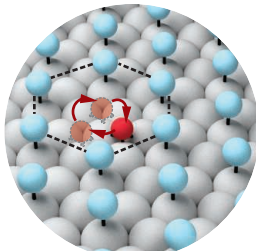
On a clean surface, an adsorbate can move around freely.



Adsorbate exchange

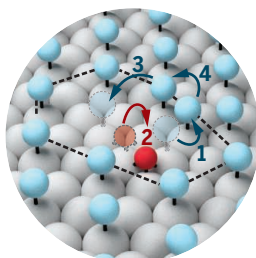
When other adsorbates are present that bind to the same positions, adsorbates move by swapping places with neighboring coadsorbates.

Movement assisted by density fluctuations



Within cage

When the adsorbate binds to a different position than the coadsorbate, it will rapidly change between equivalent sites within a cage of surrounding coadsorbates.



Cage opening

Density fluctuations allow it to break out of this cage. Diffusion then can be almost as rapid as on the clean surface.

Henß *et al.* explain this observation with a mechanism in which first one CO steps aside, moving closer to the neighboring CO adsorbates. This opens a door in the cage by which the oxygen atom can escape to an adjacent site, followed by CO rearrangement to restore the coadsorbate lattice (see the figure, bottom). Density functional calculations show this door opening to be energetically preferred both to a mechanism in which the oxygen atom jumps first and triggers the necessary CO displacement and to a concerted ringlike exchange of O and CO adsorbates.

In this scenario, the coadsorbates do not necessarily represent static obstacles to adsorbate diffusion, as implied in the site blocking concept. Rather, natural dynamic fluctuations in the density of the coadsorbate lattice can enable efficient transport pathways for the embedded diffusing species. One might expect the surrounding coadsorbate layer to nevertheless still lead to a strong reduction in the diffusion rate. The reason why this is not the case in the system studied by Henß *et al.* is probably that although the CO adlayer is ordered, its density is only 50% of the saturation value. This facilitates the fluctuations required for this mechanism. Future studies should clarify the effect of adlayer density and in-plane order. In addition to the dynamic effects highlighted by Henß *et al.*, coadsorbed layers may also weaken or modify the adsorbate's binding to the surface and thus lower the energy barriers that result from the adsorbate-surface interaction or even change the diffusion mechanism.

Henß *et al.*'s findings show that the influence of coadsorbates on adsorbate diffusion are complex and do not necessarily result in a reduction of the surface mobility. This has important consequences for understanding real-world interface processes. In the field of interface reactions, such as in heterogeneous catalysis, high mobility ensures rapid intermixing of the species on the surface. As pointed out by Henß *et al.*, this rapid intermixing is of substantial relevance for macroscopic reaction kinetics. Fast diffusion is also important for mineralization, technological deposition, and organic self-assembly. With better understanding of the way by which coadsorbates control surface transport, these processes may be fine-tuned through the targeted development of suitable additives. ■

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GEOPHYSICS

Earth's rugged lower mantle

Seismic data reveal kilometer-scale topography of the lower-mantle boundary

By Christine Houser

To know a planet is to know its boundaries, where rapid changes in state and/or composition occur. The rock-atmosphere boundary is the one we surface dwellers are most familiar with, but other boundaries lie hidden deep within Earth; for example, the crust-mantle boundary is a change from more silicon-rich rock to denser, more magnesium-rich rock. The transport of heat and rock between the upper and lower mantle largely determines the evolution of our planet, but little is known about this boundary at small scales. On page 736 of this issue, Wu *et al.* (1) report seismic-array data that suggest the existence of 1- to 3-km ripples along the top of the lower mantle. Such a structure can only be maintained across boundaries with distinct chemistry, indicating that portions of the lower mantle may contain distinct relics from the planet's earliest history.

Popular representations of the inner Earth depict the mantle as a yellow or red substance that appears to ooze across the planet. Much to the contrary, the upper mantle is a solid rock that is mostly greenish in color. It largely consists of a 60/40 blend of olivine (also known as peridot) and pyroxene. The properties of these minerals change substantially as temperatures and pressures rise with depth. Beginning at ~1800 K and 400 km depth (22 GPa), changes in the olivine mineral structure increase the mantle's rigidity and density, leading to seismic wave reflections; at a similar depth, pyroxene gradually changes to garnet phases. This region is referred to as the mantle transition zone.

At ~1900 K and 660 km depth (26 GPa), the garnet and olivine phases rapidly convert to perovskite [now called bridgmanite (2)], magnesium-iron oxide, and calcium perovskite, which persist all the way to the mantle-core boundary. This rapid change in mineralogy is accompanied by an ~30-fold

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increase in viscosity. This region marks the transition from upper to lower mantle and is critical to the mixing between the upper mantle [from which we have hand samples (3)] and the lower mantle [from which we have little but a few diamond inclusions (4, 5)]. The consensus (6) is that 80% or more of the lower mantle is the high-viscosity bridgmanite phase, with around 17% of the weaker magnesium-iron oxide and the remainder as calcium perovskite.

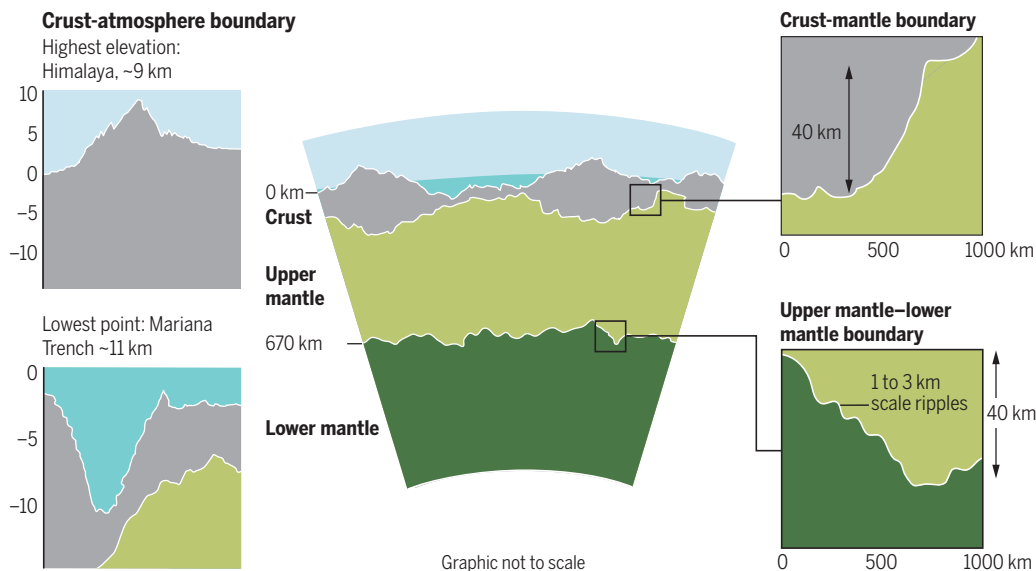
We rely on the reverberations of seismic waves to detect Earth's layers and their properties. Every earthquake with a magnitude greater than about 5.0 floods the planet with seismic energy that sloshes back and forth for hours afterward in the form of body waves [those that travel through the planet, divided into compressional (*P*) waves and shear (*S*) waves] and surface waves (those that roll along the surface). These movements amount to only micrometers at Earth's surface, requiring sensitive seismometers to measure and record these motions. Seismic waves radiate at wavelengths from a few meters to thousands of kilometers. But the lowest-noise seismic records occur at long (~100 km) and short (~5 km) wavelengths. Therefore, seismologists typically examine Earth at short scales or long scales.

The dramatic change in mineralogy from the upper to the lower mantle results in a small increase in wave speed and density (7), so that each time a wave passes through Earth, a small amount of energy is reflected or converted from shear to compression and vice versa. By combining many seismic records, scientists have shown that these reflections and conversions reveal the boundary between the upper and lower mantle to occur at around 660 km depth, with topography that undulates by 30 to 40 km over scales of hundreds to thousands of kilometers. It has not been possible to map the 660-km seismic discontinuity at scales of a few kilometers owing to a lack of coherent signals at these wavelengths.

Wu *et al.* now obtain data suggesting such topography by exploiting that in places where seismometers or seismic arrays are within ~4000 km of strong earthquakes (around magnitude 7.0), it is possible to record *P* waves that have traveled through the core, to the other side of the planet, and back again. These waves are known in

The topography of Earth's boundaries

Seismic data are beginning to reveal the large-scale topography of the boundaries between Earth's crust and mantle and between the upper and the lower mantle. The height of these large-scale topographies resembles that at Earth's surface. Wu *et al.* now report evidence for smaller, kilometer-scale topography at the upper–lower mantle boundary.



body wave seismology as *PKPPKP*, which is abbreviated to *P'P'*. Any reflected energy along the way at a depth *d* is then known as *P'dP'*. If the boundary is smooth, all reflected energy will arrive at the same time, creating a peak in the stacked seismic data. However, if the surface is rough, the energy will be spread out, instead creating a plateau. Using carefully selected earthquakes and stations, Wu *et al.* have gleamed a plateau of energy from *P'660P'*, indicating rough topography of 1 to 3 km.

“The transport...between the upper and lower mantle largely determines the evolution of our planet, but little is known about this boundary at small scales.”

The results may help to answer fundamental questions about Earth's evolution. Geologists estimate Earth's composition on the basis of what we can see from the crust and small samples of the upper mantle and what we can infer, such as a mostly iron core. However, comparing Earth with the Sun and other bodies in the solar system, there are discrepancies in bulk element abundance and isotopic ratios. Whether these discrepancies are due to sequestration of rock early in Earth's history or to heterogeneities in the protoplanetary disk remains a fundamental question that connects planet formation to the current Earth structure.

Wu *et al.*'s findings suggest that answers may come from the lower mantle. The lower-mantle topography at long (thousands of kilometers) and short (a few kilometers) wavelengths is analogous to the type of surface topography we observe on Earth (see the figure) and other planets, suggesting that in some regions, the lower mantle is chemically distinct from the upper mantle above it. Thus, these distinct regions differ in the extent to which they have mixed with the rest of the mantle since Earth's formation, presumably with the sluggish lower mantle resisting convection. Three-dimensional rendering of seismically fast and slow regions reveals the subduction of oceanic crust and tectonic plates into the lower mantle and warm upwelling regions returning material back to the upper mantle (8). But it appears that the lower mantle is also a vault-preserving relic of the time when Earth emerged from dust to become a card-carrying planet. ■

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10.1126/science.aaw4601

RETROSPECTIVE

Roy Glauber (1925–2018)

Father of quantum optics

By **Marlan O. Scully**^{1,2,3} and
Sudhakar Prasad^{4,5}

Roy Glauber, theoretical physicist and recipient of half of the 2005 Nobel Prize in Physics, passed away on 26 December 2018. He was 93. Roy was universally revered as a pioneer of the fields of nuclear scattering and quantum optics. His early elucidation of the quantum mechanical underpinnings of optical coherence formed the basis for advances in laser physics and quantum optics. His optical approximations to high-energy nuclear scattering still serve as the starting point of more detailed analyses of such scattering phenomena.

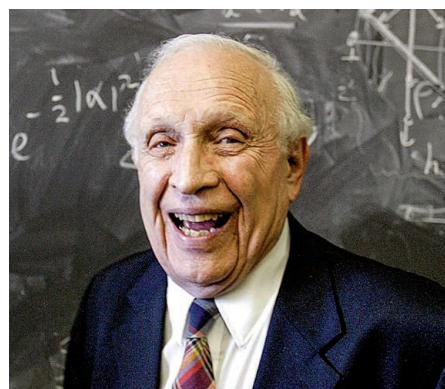
Roy was a child prodigy. In his early years, he made a working reflection telescope and, after learning that the spectra of starlight were key to further understanding stars, a spectrometer. He graduated from the famous Bronx School of Science high school in 1941 at age 16. Although he had originally planned to attend Rensselaer Polytechnic Institute and major in engineering, a full scholarship persuaded him to attend Harvard University instead.

His undergraduate years were dramatically altered by the Japanese attack on Pearl Harbor. The faculty began leaving Harvard for wartime positions, and Roy was invited to work on the Manhattan Project, at just 18 years of age. He spent 2 years in Los Alamos in the company of esteemed physicists such as John von Neumann, Enrico Fermi, Niels Bohr, Hans Bethe, and J. Robert Oppenheimer. For the Trinity test (the first nuclear detonation), Roy and some of his young colleagues climbed a nearby mountain in the hopes of seeing the event. They had already begun to pack up, assuming that they had missed it or that it hadn't worked, when the southern sky lit up. The image was indelibly burned into Roy's memory.

Roy then returned to Harvard and joined theoretical physicist Julian Schwinger's group. Although he never published with Schwinger, the aspects of coherent meson-

pion physics he learned while working with him strongly influenced Roy's later work on the photon coherent state. After earning his bachelor's degree in 1946 and his Ph.D. in 1949, both from Harvard, Roy spent 6 months in Zürich, Switzerland, with the famous theoretical physicist Wolfgang Pauli, and then accepted an invitation from Oppenheimer to work at the Institute for Advanced Study in Princeton, New Jersey.

Next, he spent a year researching the electron diffraction of molecules in the Caltech chemistry department under Linus Pauling. There, he developed an accurate approximation of this problem by adapting the more familiar methods of optical diffraction. He realized that his approach to nuclear scattering analysis would become increasingly important as improved particle-accelerator design enabled higher particle energies. He left Caltech to join the faculty at Harvard, where he continued his studies of nuclear scattering over the next decade. The well-known Glauber-eikonal theory of high-energy nuclear



scattering that eventually emerged from this work is still used to treat high-energy collisions of heavy nuclei as a probe of their detailed structure.

After the first experiments of Hanbury Brown and Twiss on intensity interferometry in 1954, Roy became interested in the higher-order field correlation functions. He produced a beautiful account of the multiphoton interference phenomena using such correlation functions, which serve as a valuable tool in quantum optics. He also authored an insightful paper on the stochastic dynamics of the kinetic Ising model, which has been used more recently to treat the nonexponential relaxation of linear-chain polymers.

In addition to Roy's outstanding physics research, he was an excellent teacher, contributing mightily to the education and understanding of generations of physicists. His 1963 papers on the quantum theory of optical coherence, which earned him the Nobel Prize, were pedagogical masterpieces; they read like a textbook. Committed to his teach-

ing, he rarely missed a class, even on the day he learned that he had won the Nobel. As a fellow of the Texas A&M University Hagler Institute for Advanced Study, he lectured widely in Texas and beyond.

Roy was a frequent participant in conferences around the world and a popular keynote speaker at many conference banquets, regaling his fellow attendees with his wry sense of humor, quirky quips, and impressive recall. His sense of humor was on full display at the Ig Nobel awards, a Nobel Prize parody that recognizes achievements in seemingly absurd (ignoble) research. As part of the annual ceremony, paper airplanes are launched en masse toward the stage. For years, Roy gleefully served as "keeper of the broom," sweeping up the piles of airplanes.

S.P. met Roy in 1978 and went on to become his last Ph.D. student. Roy was a demanding and inspirational mentor. A knock on his door would be met with his signature cough, followed by his clarion voice summoning his visitor. He insisted that his students acquire a broad foundation of physics before thinking about research, and he required them to think clearly and express their points with clarity. When researchers suggested that they may have settled on a solution to a question, he would find another important aspect of the problem for them to explore. Roy was rarely persuaded that a manuscript was ready until he had thoroughly vetted it for rigor and clarity, a process that could take months.

M.O.S. was fortunate to have Roy as a mentor and friend for his entire career. They often hiked together. On one outing, they encountered a rattlesnake and turned it into a hatband. On another, they saw a school of fish, and Roy joked that the fish obeyed the Poisson statistics of coherent light. Every experience was more fun with Roy.

Roy was a dedicated father who raised his children, Jeff and Valerie, as a single parent. He admitted without regret that he had traded some of his scientific research pursuits for the rewards of fatherhood. Roy's partner in later years, Atholie Rosett, accompanied him to Sweden for the Nobel festivities in 2005. We of Roy's extended family regard her affectionately and with much appreciation for making Roy's later years pleasant and productive.

Roy retired from Harvard in 2012, took up a position at Texas A&M, and continued to enjoy traveling to conferences around the world. He remained active and lucid to the very end. A veritable giant of modern theoretical physics and a teacher at his very core, he will continue to enrich the lives of generations of physicists and students to come. His legacy as a scientist, educator, father, and friend lives on. ■

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Band-e Amir's six sapphire-blue lakes became Afghanistan's first national park in 2009.

BOOKS *et al.*

WILDLIFE CONSERVATION

Conservation and conflict

Afghanistan's rich natural heritage shines in an insider's account of the foundings of its first national parks

By **Rosie Cooney**¹ and **Khalil Karimov**²

In war zones—where daily life holds existential threats, where people are strafed and hardened by conflict, where securing food and safety is an ever-present priority—why should people care about the elusive beauty of the snow leopard, the hoary majesty of the markhor goat, or the fantastically adorned Marco Polo sheep? Alex Dehgan tackles this question in his insightful and eye-opening book, *The Snow Leopard Project*, a narrative of his experiences establishing an outpost of the Wildlife Conservation Society (WCS)—one of the leading conservation organizations in the United States—in Afghanistan in 2006.

From the outset, Dehgan and his colleagues grappled with unpredictable situations and jaw-dropping logistic and security challenges: from dodging IEDs (improvised explosive devices) to adjusting camera trap schedules to accommodate U.S. bombing campaigns. What appears to have been the biggest challenge, however, was navigating the priorities and processes of the U.S.

Agency for International Development, the project's major financial backer.

Central Asia, for most readers, is likely to be a black zone on the map—a geographical lacuna that may, if anything, conjure the Silk Road, Alexander the Great, brutal conflicts, or post-Communist corruption. Yet, as Dehgan highlights, this area is rich, diverse, and valuable in both cultural and biological terms. Peoples have moved and settled across the region over millennia, making a complex cultural mosaic. And the mountain ranges around the north of Afghanistan and its bordering countries represent the convergence zone of the Afrotropic, Indomalayan, and Palearctic faunas, making for a distinct and rich wildlife assemblage.

Dehgan's writing is filled with love and respect and an interest in Afghanistan and the broader region—people as well as wildlife. The book is filled with characters, Afghans and expats, drawn with affection and detail. An opening anecdote about bird-watching with a member of the Taliban illustrates neatly his ability to build personal bonds in support of conservation.

There are themes here that resonate deeply with conservation efforts worldwide. It is important, for example, to gain the support of local people and to build effective governance structures for protected areas that give these

people a voice and ensure that they gain tangible benefits from conservation efforts. The political challenges of mobilizing cooperation between states in conflict, in which wild species and landscapes rank at the bottom of a long list of priorities, will likewise be familiar to conservationists in other regions.

Despite this region's challenges—the land mines, the devastated infrastructure, the shattered society, the yawning chasms of governance—this is a book with a message of hope. Dehgan found enthusiasm and support for wildlife conservation among the people of Afghanistan at all levels, from political leaders to ordinary rural people. He attributes this to a desire to reestablish the country's identity after decades of war and displacement. The species that will benefit from these efforts—the urial, the golden jackal, the musk deer, and the eponymous snow leopard, for example—are an integral part of the country's natural heritage, deeply interwoven with their culture (the vast majority of Afghans are directly dependent on natural resources). Wildlife recovery reflects the potential for rebounding national identity and pride.

This is indeed a story of success (albeit partial and hard-won). WCS's work helped Afghanistan establish its first two national parks: Band-e-Amir, in the central highlands, in 2009, and Wakhan, in the high and remote northeast, in 2014. Although ecotourism in Afghanistan may seem a hard sell, domestic and international interest is growing; the parks have been developing infrastructure and training to accommodate tourists, and local benefits have started to flow. Dehgan notes that in 2017, 189,000 people (mainly Afghan nationals) visited Band-e-Amir.

The Snow Leopard Project is not without flaws. It is somewhat weak in narrative tension; Dehgan's account is essentially a collection of anecdotes loosely held together with personal reflections. We never learn enough about the writer himself to be caught up in a compelling overarching story, and the book abruptly ends with his resignation from the WCS, with little insight into the precipitating dynamics or his feelings on the matter.

Overall, however, the book abundantly succeeds in highlighting the stark and surprising challenges faced by those engaged in conservation in war zones and in shining a light on the rich cultural and biological diversity of Afghanistan. The country (aided by WCS and others) has taken the first steps toward recovering its wildlife and related cultures and livelihoods; with additional support from the global community—including tourists and donors—great things are possible. ■

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The Snow Leopard Project
Alex Dehgan
PublicAffairs, 2019.
288 pp.

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HISTORY OF MEDICINE

Overlooked no longer

A new play honors the enslaved black women who helped improve our understanding of reproductive health

By **Deirdre Cooper Owens**

In the era of #metoo, #citeblackwomen, and #blacklivesmatter—all movements founded by black women that, collectively, seek to call attention to pervasive social injustices and secure acknowledgment of this group's contributions to American society—Charly Evon Simpson has written a play that centers on the experiences of antebellum-era enslaved women, whose sufferings provided a platform for white medical men to pioneer surgical techniques.

As I waited for the play to begin, I was moved by the starkness and simplicity of the stage design. There was an ever-present haze that evoked for me the often obscured nature of slavery in this country's collective imagination. Most Americans connect slavery and the slave's work to plantations and cotton. However, it was clear that Simpson and director Colette Robert wanted to convey that enslaved people's labor, especially enslaved women's labor, was also reproductive.

James Marion Sims is the historical subject whose controversial gynecological experiments on enslaved women led to a successful surgical repair for obstetrical fistulae

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Behind the Sheet

Charly Evon Simpson

The Ensemble Studio Theatre, New York, NY, USA.

patients and who inspired Simpson to write the play. Simpson, however, chooses to tell the story from the perspective of the women.

Philomena, the main character, played effectively by Naomi Lorrain, emerges as the predominant voice. It is she who instructs the other enslaved women on the nature of their illnesses, naming and defining the medical terms that “Dr. George”

has written in his notebook. Most audiences would have no trouble inferring the meaning of these terms from contextual clues in the dialogue; however, Simpson's positioning of Philomena as a learned slave who is able to code switch with ease was an effective way to encourage viewers to think

about enslaved people as human beings and not as unsophisticated property.

Philomena, who is George's assistant and sex slave, is an ever-present voice of compassion and contestation. She insists that his patients experience pain during the surgeries he subjects them to, despite prevailing scientific thought, which held that black women experienced no pain during childbirth and very little pain from operations. And she reminds the audience that the lived experiences of black women should be heard and believed. At times, her speeches are heavy-handed, but alas, slavery itself was heavy-handed.

As the rest of the cast—a core group of enslaved women (Betty, Sally, Mary, and Dinah) and a lone enslaved man (Lewis)—emerge,

the audience sees how enslaved people had to suppress their own challenges, feelings, and fears in order to mitigate those of the men and women who owned them. Lewis, for example, is played brilliantly by Shawn Randall, who shows the full range of human frailty and strength. His portrayal conjured up how enslaved people toed a thin line where both sides were fraught: Either they acquiesced to ever-increasing subjugation from their owners or fought back, risking the repercussions of the owners' emotional volatility.

Although their roles were secondary, Stephen James Anthony's depiction of Samuel, George's white male assistant, and Megan Tusing's role as George's wife, Josephine, brought life to characters who at times slipped into flattened tropes of whiteness. Through these characters, the audience sees how slavery helped to construct ideas about whiteness and gender during the 19th century. They are self-absorbed, to be sure, but their self-absorption is about their own lack of agency in the world in which they live, a world that created and reinforced structures that upheld advanced age, masculinity, biological whiteness, and ownership of property as the hallmarks of citizenship and worthiness. The penultimate scene, in which George and Josephine discuss his absence from his family's life because of his career ambitions and demands, was acted beautifully, conveying the sincere longing and vulnerability that Tusing brought to her character.

I wish that the same nuance had been brought to the enslaved women's roles. These characters at times seemed like stock depictions of the sassy slave (Sally), the haughty house slave (Betty), the refined concubine (Philomena), the angry black woman (Dinah), and the naïve and sensitive slave (Mary). In the final ensemble scene, however, the women reveal vulnerabilities and express love to each other in ways that showed their lives and bodies matter too. This ending was one of the more powerfully acted scenes in the play, and it was in these moments of emotional vulnerability that the actors shone.

Watching a play about slavery, rape, racism, and experimental medicine is difficult. I'm not sure if one could emerge from the experience having “enjoyed” it. Although there were moments when I grew frustrated at the heavy-handed dialogue, the sometimes flat character depictions, and the inelegant data dumps, Simpson brought sensitivity and honor to enslaved black women's lives, revealing how their suffering sparked innovation that has improved the reproductive health of all women. I look forward to her continued growth as a playwright and as an important new voice in American theater. ■

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Cristina Pitter, Naomi Lorrain, and Nia Calloway portray Sally, Philomena, and Betty, respectively.



LETTERS

Edited by **Jennifer Sills**

Saving China's onager

The onager (*Equus hemionus*), a state-protected species in China, is listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (1) and categorized as near threatened by the International Union for Conservation of Nature (IUCN) (2). The main threats to the species include habitat loss caused by human activity, excessive hunting for food and trade, resource competition, climate change, disease, genetic stochasticity, and inbreeding (3–6). In recent decades, coal resource development in China has grown, adding to the threats faced by the remaining onager populations.

The northeastern part of the Junggar Basin, where most onagers live (1), is also home to abundant coal reserves and China's largest integrated coalfield. To meet the needs of the coal industry, the local government reduced the Kalamaili Mountain Ungulate Nature Reserve, the most important refuge of onagers in China, to 71% of its original size (7). The accommodations made for the coal industry have reduced the size of onager habitats, blocked access to drinking water, and disturbed them with the noise generated by the development. Meanwhile, railway construction required for the development of the coal industry has obstructed migration routes (6, 8, 9).

China's onagers are an important genetic resource for breeding new onager

species, an effort that could protect species diversity, genetic diversity, and ecological diversity (10). Although the Chinese government has built a nature reserve to protect onagers, public awareness of wildlife protection remains low. To ensure the safety of China's onagers, China must further expand the current nature reserves and implement scientific management and protection policies. The government should also better publicize wildlife protection.

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Hemp hemp hooray for cannabis research

In December 2018, the U.S. Congress passed the Agriculture Improvement Act of 2018, better known as the farm bill, which redefines hemp as any part of the

China's onager (*Equus hemionus*) and its habitats are endangered by coal resource development.

Cannabis plant with a tetrahydrocannabinol (THC) concentration of less than 0.3% (1). Although any *Cannabis* plant with a THC content greater than 0.3% remains classified as a schedule I drug, making it difficult to access, researchers can take advantage of the revised hemp definition to better understand medicinal, agricultural, biological, ecological, and industrial properties of the *Cannabis* plant.

Before the farm bill passed, the inability to procure suitable material in the United States limited *Cannabis* drug research (2). Now, scientists armed with appropriate state or U.S. Department of Agriculture licenses can grow or purchase materials needed for studies involving most cannabinoids. This allows scientists to investigate properties of the rapidly diversifying *Cannabis* varieties and products not readily available through the government. Recently, the U.S. Food and Drug Administration approved a cannabidiol (CBD)-based drug for the treatment of two forms of childhood epilepsy (3). Preliminary research indicates that CBD may also be effective as a treatment for anxiety, cancer, depression, inflammation, pain, and neurodegenerative diseases (4, 5).

The farm bill's new definition of hemp will allow researchers to order live seeds without limitation, facilitating biological and agricultural research. For example, unlike the seeds of many commercial crops (such as corn and wheat), hemp seeds are attractive to many animals (6). Moreover,

hemp requires limited agricultural inputs in fields, thus allowing greater biodiversity than that supported by a field of conventional crops (7). Hemp can also bioremediate toxins from the environment (8).

Researchers studying industrial applications will also now have better access to hemp products. Hemp fiber is environmentally friendly to produce and has a high strength-to-weight ratio (9). Given their properties, hempseed oil, fiber, and the inner woody core of the stem may be valuable for developing sustainable construction materials that sequester carbon, such as epoxies, biocomposite plastics, and hempcrete, respectively (10). Hemp fibers may also be a cheap platform for producing carbon nanosheets for electronics applications (11). Furthermore, the fast growth, high yield, and cellulose-rich content of hemp stalks provide a suitable platform for biofuel production (12). Until now, the cost of importing hemp into the United States has hindered development of such applications. Passage of the farm bill provides exciting opportunities to explore new uses of hemp and the development of sustainable products.

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Argentina's subpar investment in science

Socioeconomic progress is closely linked to investment in research and development (R&D) (1). Countries can encourage economic growth by spending a substantial share of gross domestic product



Hempseed oil, now more accessible to researchers, could be used to develop sustainable epoxies.

(GDP) on R&D (2). Argentina, which is in the midst of an economic crisis that is hitting scientists hard ("Argentina's scientists engulfed in budget crisis," E. Rodríguez Mega, News, 16 November 2018, <https://scim.ag/ArgentinaBudget>), has not noticeably increased research spending in the past decade (3), despite promises to invest more (4, 5). In 2015, Argentina invested 0.63% GDP into R&D, compared with Brazil's 1.28% and 2.74% by the United States (3). Argentina's science enterprise also suffers because 80% of the country's R&D funding comes from the public sector, unlike in Brazil and the United States, where the private sector contributes substantially (6). To spur scientific progress and economic growth, Argentina's government should implement policies that increase R&D funding.

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Limited funding impairs performance, especially for early-career researchers, who comprise an energetic, highly skilled, and mobile labor force (7). Argentina should nurture young investigators by encouraging international training, incentivizing their return upon its completion, and providing grants that are competitive with those available in other countries. Currently, initiation grants in Argentina are substantially lower than those in the United States. For example, Argentina's 2018 PICT-I-D grant ranged from about US\$3,500 to US\$5,000 per year (8), whereas the 2018 U.S. K99 was US\$113,000 in 2018 (9). Although researchers are often encouraged to resort to external sources of funding, the agendas of international organizations are not necessarily aligned with the domestic needs (10).

Policy-makers in Argentina should also facilitate an increase in private-sector R&D funding, which would increase the proportion of GDP devoted to R&D without requiring additional public spending. Finally, Argentina's government could decrease research costs and facilitate the acquisition of materials. Imported reagents and supplies are subjected to substantial tariffs and delays due to customs (10). The government should provide tax breaks and work to expedite their delivery to labs.

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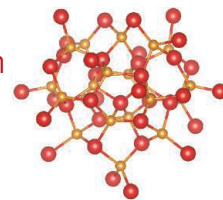
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RESEARCH

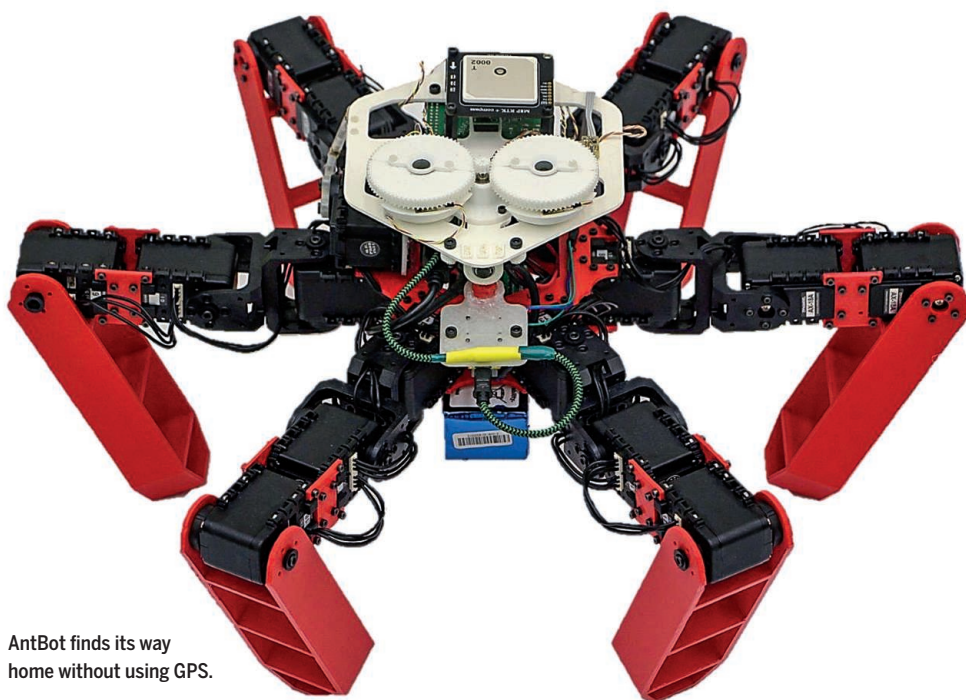
Reversible isomerization in magic-size nanoclusters

Williamson et al., p. 731



IN SCIENCE JOURNALS

Edited by **Stella Hurtley**



AntBot finds its way home without using GPS.

ROBOTIC NAVIGATION

There's no place like home

GPS is not always available, let alone perfect. Dupeyroux *et al.* created the six-legged AntBot, which integrated multiple data sources—but not GPS—to track its position. This was inspired by the multisensory homing method of desert ants, which does not use pheromones because of the destructive desert heat. After a random walk, AntBot went directly back to its starting point by combining tracked distance with directional data from two sensors that polarized and detected light. AntBot is sensitive to ultraviolet light, which means it can get home even under a cloudy sky. —RLK

Sci. Robot. **4**, eaau0307 (2019).

IMMUNOLOGY

B1 or B2? The BCR decides

Immunological B cells are generally divided into two major subsets. B2 cells generate specific antibodies against foreign antigens in secondary lymphoid organs. B1 cells, found predominantly in the peritoneal and pleural cavities, instead produce “natural” antibodies as part of the innate immune system. Two models to explain this split exist: the “lineage model,” wherein both subsets have distinct progenitors, and the “selection model,” in which fates are directed by different B cell antigen receptors (BCRs). Graf *et al.* provide support for the selection model using a transgenic system in which BCR

specificities can be changed. Mature B2 cells differentiated into functional B1 cells when a self-reactive B1 BCR was swapped in, in the absence of B1 lineage precommitment. —STS

Science, this issue p. 748

CERAMICS

Elastic ceramics

Aerogels hold promise as lightweight replacements for thermal insulation. However, poor mechanical stability has hampered progress in moving toward commercialization. Xu *et al.* designed a mechanical metamaterial that pinches in a small amount when you compress it (see the Perspective by Chhowalla and Jariwala). This is characteristic of materials with a negative Poisson's ratio

and dramatically improves mechanical stability. The trick was using three-dimensional graphene structures to template the ceramic aerogels, thus producing a superinsulating material endowed with excellent mechanical properties. —BG

Science, this issue p. 723;
see also p. 694

SURFACE SCIENCE

A path through a crowd

Catalytic reactions on surfaces occur at pressures at which the surfaces are completely covered with adsorbed molecules. It would seem that this arrangement would interfere with reactants encountering one another through diffusion processes. Henß *et al.* used

high-speed scanning tunneling microscopy to follow the diffusion of oxygen atoms on a rubidium surface that was fully covered with carbon monoxide (CO) molecules (see the Perspective by Magnussen). Oxygen-atom diffusion was unexpectedly fast. A theoretical model revealed that CO diffusion appears to open pathways for oxygen-atom movement. —PDS

Science, this issue p. 715;
see also p. 695

DEVICE TECHNOLOGY

Low-power organic transistors

For internet-of-things applications, transistors that deliver high signal amplification (high

gain) at low power will help conserve power and extend battery life. Jiang *et al.* used inkjet printing to fabricate an organic transistor in which silver metal contacts form a low Schottky barrier (less than 0.2 electron volt) with an organic semiconductor. The transistor delivered gain near the theoretical limit at a power below 1 nanowatt and detected electrophysiological signals from the skin with a wearable device. —PDS

Science, this issue p. 719

QUANTUM OPTICS

An integrated route to quantum detection

The quantum properties of the nitrogen-vacancy (NV) center defect in diamond are being pursued as building blocks for quantum-enhanced technologies. Addressing and manipulating the defects, however, typically requires bulk optics, which could limit scalability. Siyushev *et al.* developed an on-chip technique in which the NV center is detected optoelectronically. Such a detection and manipulation method offers a route to develop an integrated platform for scalable quantum-based sensing technologies. —ISO

Science, this issue p. 728

STRUCTURAL BIOLOGY

Mechanism of ribosome rescue

Bacterial ribosomes that stall on truncated or cleaved messenger RNA (mRNA) are rescued by trans-translation. Two factors, transfer-messenger RNA (tmRNA) and small protein B (SmpB), resolve the stalled complex by tagging the nascent polypeptide for degradation and facilitating release of the ribosome. Rae *et al.* determined structures of key trans-translation intermediates. The structures reveal how SmpB identifies stalled ribosomes; how the large, circularized tmRNA molecule moves through the ribosome;

and how translation is shifted from the truncated mRNA to tmRNA. —SYM

Science, this issue p. 740

STRUCTURAL BIOLOGY

To transport or not to transport

Therapeutic drug delivery into cells is complicated by membrane proteins like ABCB1 (also termed P-glycoprotein) that shuttle diverse compounds out of cells. Alam *et al.* determined high-resolution cryo-electron microscopy structures of ABCB1 bound either to a substrate, the cancer drug Taxol, or to the ABCB1 inhibitor zosuquidar. The conformational changes that facilitate drug transport are caused by hydrolysis of adenosine triphosphate (ATP). The structures show that, although Taxol and zosuquidar bind to the same site, subtle structural differences lead to altered conformations of the nucleotide binding domains that are responsible for ATP hydrolysis. —VV

Science, this issue p. 753

PAIN MEDICATIONS

Toward a painkilling nanomedicine

America's opioid epidemic has resulted in large-scale initiatives to identify opioid substitutes. However, for many cases of chronic pain, no viable alternatives to opioids exist. In an effort to expand the arsenal of antipain treatments, Feng *et al.* bonded Leu-enkephalin with the lipid squalene. Enkephalins, like endorphins, are naturally occurring peptides in the human brain. They act on the opioid receptors to manage pain but have proved difficult to exploit therapeutically. When incorporated into nanoparticles with squalene, Leu-enkephalin exhibited a more controlled release that localized in inflamed tissue, which is promising news for nonopioid pain treatment. —KJP

Sci. Adv. 10.1126/sciadv.aau5148 (2019).

IN OTHER JOURNALS

Edited by **Caroline Ash**
and **Jesse Smith**



CELL BIOLOGY

Chromosome axis organization

The chromosome axis is a meiosis-specific structure that is essential for proper chromosome pairing and meiotic recombination. It is conserved among all eukaryotes; however, the key axis protein components are evolutionarily divergent among different species. West *et al.* characterized axis proteins in baking yeast, a mustard plant, and human. Although these proteins have little sequence homology, they all form filaments from tetrameric units and recruit key players that mediate downstream meiotic recombination. This common assembly feature ensures that the architecture of the meiotic-chromosome axis is highly conserved across fungi, mammals, and plants. —SYM

eLife 8, e40372 (2019).

CELL BIOLOGY

Tunneling nanotubes under the microscope

Long, actin-rich membranous protrusions called tunneling

nanotubes (TNTs) allow the intercellular transport of various cargos, including viruses, organelles, and proteins. Sartori-Rupp *et al.* report the structural characterization of TNTs formed between neuronal cells in culture using correlative light- and cryo-electron microscopy approaches. They found that TNTs are distinct from other actin-rich cell protrusions like filopodia. TNTs are composed of a bundle of functional individual tunneling nanotubes containing membrane-bound compartments, including mitochondria. Bridging threads between the individual nanotubes contained the cell adhesion molecule N-cadherin. —SMH

Nat. Commun. 10, 342 (2019).

ORGANIC CHEMISTRY

Illuminating a path uphill to open rings

When a chemical compound absorbs light directly, it gets a burst of energy that can propel an otherwise unfavorable reaction forward. In principle, light-absorbing catalysts can likewise channel energy to substrates to push reactions uphill

PHOTO: ISTOCK.COM/STEPHANE NOIRET



NEUROSCIENCE

Sleeping in standby mode

Sleep is essential, but it makes us unable to interact and vulnerable. Yet the sleeping brain continues to process stimuli from the environment. Legendre *et al.* presented awake and sleeping subjects with relevant and irrelevant stories via both ears while recording the neural responses in their brains with electroencephalography. Although sleepers seemed unresponsive, their brains clearly registered external stimuli to an extent that depended on specific brain rhythms and sleep depth. Thus, sleepers can process surrounding events sufficiently to know when it might be a good idea to rapidly wake up. —EACP

Nat. Hum. Behav.

10.1038/s41562-018-0502-5 (2019).

Even when asleep, humans remain alert to external stimuli.

thermodynamically. Nonetheless, the recent surge in photoredox catalysis has largely focused on accelerating favorable transformations. Ota *et al.* demonstrate that a catalyst system composed of an iridium photoredox chromophore, a phosphate base, and a thiol for hydrogen-atom transfer can isomerize cyclic alcohols to higher-energy linear aldehydes. The high-yielding protocol is compatible with a wide variety of complex substrates. —JSY

J. Am. Chem. Soc. **141**, 1457 (2019).

ALZHEIMER'S DISEASE

Targeting Tau

Cholesterol metabolism is linked to Alzheimer's disease (AD) pathogenesis; however, the pathways involved are only partially understood. Van der Kant *et al.* associated cholesterol with the accumulation of phosphorylated Tau (pTau) protein in neurons, a hallmark of AD. The study screened compounds for the ability to block pTau accumulation in neurons that were derived from AD patients. Drugs that decreased cholesteryl esters (CEs) also reduced pTau. The effective drugs included

statins, which block cholesterol synthesis, and drugs that alter the metabolism of cholesterol into CEs or 24-hydroxycholesterol. Reducing neuronal CEs was associated with increased proteasome-mediated degradation of pTau. —LC

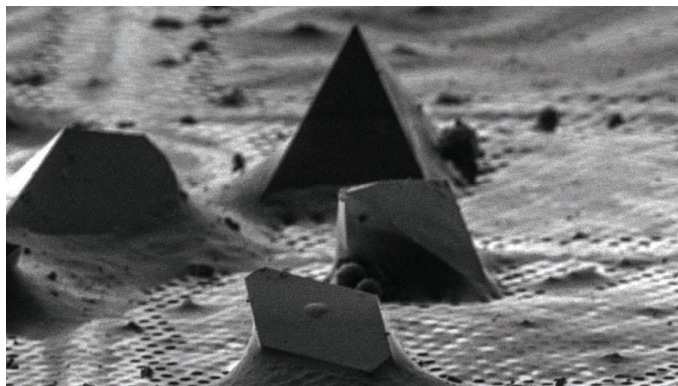
Cell Stem Cell 10.1016/j.stem.2018.12.013 (2018).

ELECTRON DIFFRACTION

Microsolution for macromolecules

Protein crystallographers who hope to use x-ray diffraction

to determine structures have long struggled with growing large, uniform protein crystals. Crystals only a few micrometers thin are ideal for diffraction by electrons but are likewise hard to grow predictably. Focused ion beam milling can create thin slices from large protein crystals that are ideal for electron diffraction. Martynowycz *et al.* demonstrate that continuous rotation of a single crystal section with careful control of electron dose can yield a high-resolution structure of a model protein. Such a strategy should be generally applicable



Macromolecular protein crystals can be prepared for microcrystal electron diffraction by using focused ion beam milling.

to otherwise intractable microcrystals, which are, as of now, a common dead end. —MAF

Structure 10.1016/j.str.2018.12.003 (2019).

ASTROCHEMISTRY

Molecules in interstellar space

Chemistry in space occurs wherever gas is dense and cool enough for chemical bonds to form, producing a wide variety of molecules. These are particularly common in the gas clouds that provide the raw material for star and planet formation. McGuire has cataloged all molecules detected in the interstellar and circumstellar medium. More than 200 distinct molecules have been found, increasing at an average rate of four or five per year. The inventory of known molecules is heavily biased toward those that are easy to observe with radio telescopes. Thousands of astronomically observed molecular lines remain unassigned, often because of a lack of laboratory spectroscopy for comparison. —KTS

Astrophys. J. Suppl. Ser. **239**, 17 (2018).

PLANT SCIENCE

Optimizing agricultural fertilization

Although potassium (K) is the seventh-most-abundant element in Earth's crust, mineable resources of bioavailable K are finite. Plants depend on K for growth and development, but most K in soil is inaccessible to them. Dhillon *et al.* calculated the K use efficiency for cereal crops worldwide over a 55-year span and found that yields in recent decades have increased faster than the addition of new land for cultivation. This is due in part to the increased use of fertilizers that include K; however, K use efficiency has not improved. What is needed is fine-scale analysis of bioavailable K in agricultural fields, attention to the soil microbiome, and avoiding oversupply of K. —PJH

Agron. J. 10.2134/agronj2018.07.0462 (2018).

ALSO IN *SCIENCE* JOURNALSEdited by **Stella Hurtley****MAGNETISM****The ultimate in thin-film magnetism**

The alignment of the magnetic properties of atoms gives rise to a wealth of simple and exotic properties that can be exploited. As the dimension of the material is reduced, such that the atoms are in a single monolayer, it was widely believed that thermal fluctuations overwhelm and prevent magnetic ordering. Gong and Zhang review the developments that have followed the recent discovery of magnetism in two-dimensional materials. Recognizing that magnetic anisotropy can be used to induce stable magnetism in atomic monolayers, they provide an overview of the materials available and the physical understanding of the effects and then discuss how these effects could be exploited for widespread practical applications. —ISO

Science, this issue p. 706**STRUCTURAL BIOLOGY****The machinery behind amyloid peptides**

β -Amyloid peptides, which are derived from amyloid precursor protein (APP), form the plaques in the brain that are characteristic of Alzheimer's disease. Zhou *et al.* report a high-resolution structure of a transmembrane segment of APP bound to human γ -secretase, the transmembrane protease that cleaves APP to give β -amyloid peptides (see the Perspective by Lichtenthaler and Güner). Disease-associated mutations within presenilin-1, the catalytic subunit of APP, likely affect how the substrate is bound and thus which peptides are generated, with some being more amyloidogenic. It may now be possible to exploit the features of substrate binding to design inhibitors. —VV

Science, this issue p. 708;
see also p. 690**NEURODEGENERATION****How dipeptide repeats cause pathology**

A repeat expansion in the chromosome 9 open reading frame 72 (*C9orf72*) gene is the most common known cause of two neurodegenerative diseases: frontotemporal dementia and amyotrophic lateral sclerosis. This expansion leads to the abnormal production of proteins of repeating dipeptides, but their contribution to disease pathogenesis remains unclear. Zhang *et al.* engineered a mouse model to study the consequences of one of these dipeptides—proline-arginine dipeptide repeat protein, poly(PR)—in the brain. They found that poly(PR) caused neuron loss as well as motor and memory impairments. These detrimental effects resulted from poly(PR)-induced perturbation of heterochromatin function, a tightly packed form of DNA that represses gene expression. —SMH

Science, this issue p. 707**TOXINS****Bacterial warhead targets DNA**

The bacterial toxin colibactin causes double-stranded DNA breaks and is associated with the occurrence of bacterially induced colorectal cancer in humans. However, isolation of colibactin is difficult, and its mode of action is poorly understood. Wilson *et al.* studied *Escherichia coli* that contain the biosynthetic gene island called *pks*, which is associated with colibactin production (see the Perspective by Bleich and Arthur). They identified the DNA adducts that resulted from incubating *pks*⁺ *E. coli* in human cells. To overcome the lack of colibactin for direct analysis, mimics of the *pks* product were synthesized. From the resulting synthetic adenine-colibactin adducts, it became evident that

alkylation via a cyclopropane “warhead” breaks the DNA strands. Similar DNA adducts were then identified in the gut epithelia of mice infected with *pks*⁺ *E. coli*. —CA

Science, this issue p. 709;
see also p. 689**NANOMATERIALS****Cluster isomerization**

Structural rearrangements at the atomic scale can range from isomerization of small molecules to solid-solid phase transformations of crystals. Williamson *et al.* show that magic-size cadmium sulfide (CdS) crystalline clusters, which are about 2 nanometers in diameter and expose a large fraction of surface atoms capped by bidentate oleate ligands, undergo a reversible isomerization. The initial α -Cd₃₇S₂₀ phase, which has a wurtzite-like crystal structure, isomerizes to β -Cd₃₇S₂₀, which has a zinc blende-like structure upon exposure to methanol, and then transforms back under vacuum. This transition is driven by distortion of the ligand shell and shifts the excitonic energy gap of the clusters. —PDS

Science, this issue p. 731**GEOPHYSICS****Inferring blocked mantle convection**

The boundaries between rocks with different physical properties in Earth's interior come from either a change in crystal structure or a change in chemical composition. Wu *et al.* examined the roughness of the boundary between Earth's upper and lower mantle, thought to form from a change in mineral structure (see the Perspective by Houser). To their surprise, in some locations, the boundary has small-scale roughness that requires some chemical difference above and below the boundary. This observation provides evidence of partially blocked mantle

circulation that leads to some chemical differences between the upper and lower mantle. —BG

Science, this issue p. 736;
see also p. 696**STRUCTURAL BIOLOGY****A human P spliceosome structure**

Splicing of some pre-messenger RNAs could be regulated by cell type-specific splicing factors. Fica *et al.* describe the cryo-electron microscopy structure of the human postcatalytic (P) spliceosome. Surprisingly, it lacks the splicing factor Prp18, which plays an essential role in exon ligation in the yeast spliceosome. Instead, a metazoan-specific splicing factor, FAM32A, compensates for Prp18 and promotes exon ligation by penetrating the active sites and directly stapling the 5' exon and the 3' splice site. These findings suggest a way to control tissue-specific alternative splicing. —SYM

Science, this issue p. 710**STRUCTURAL BIOLOGY****Getting over nucleosomal barriers**

In eukaryotic cells, RNA polymerase II (RNAPII) transcribes DNA within nucleosome-coated chromatin. The nucleosomes can provide major roadblocks for transcription. Cells solve this problem by using transcription elongation factors. Ehara *et al.* solved the cryo-electron microscopy structures of the nucleosome-transcribing RNAPII with elongation factors Elf1 and Spt4/5. Elf1 and Spt4/5 cooperatively suppress RNAPII pausing at multiple super helical locations [SHL(−6), SHL(−5), and SHL(−2)] and facilitate RNAPII progression through SHL(−1) by adjusting the nucleosome position to favor forward progression. —SYM

Science, this issue p. 744

NEUROSCIENCE

Learning like a human

The deep network systems underlying artificial intelligence (AI) reflect a highly simplified version of our current understanding of human brain circuitry and the hierarchy of cellular connections. This has allowed the generation of impressive AI systems, but a major challenge remains for human-like learning and perception. In a Perspective, Ullman discusses whether more lessons can be applied from our understanding of how the brain works to improve AI. —GKA

Science, this issue p. 692

CAR T CELLS

The long and short of CAR activation

Immunological B cell malignancies can be therapeutically targeted by the adoptive transfer of T cells that express a chimeric antigen receptor (CAR). Ramello *et al.* used proteomics to understand how CARs with distinct intracellular domains activated signaling in human T cells. Independently of specific signaling domains, the overall length of CAR intracellular activation domains determined whether a CAR promoted strong T cell signaling. These data may explain why some CARs can stimulate antigen-independent tonic signaling, which leads to progressive CAR T cell inactivation. —ERW

Sci. Signal. **12**, eaap9777 (2019).

ASTHMA

Smoothing out muscle in asthma

Asthma is often treated with drugs that reduce airway inflammation. Saunders *et al.* now show that fevipiprant, a prostaglandin D₂ type 2 receptor antagonist, reduces smooth muscle mass in bronchial biopsies from asthma patients. Computational simulations of an asthmatic airway predicted that decreasing airway smooth muscle mass was necessary for

the fevipiprant-mediated amelioration of symptoms in asthma patients observed in a prior clinical trial. Treating bronchial biopsies from asthma patients with fevipiprant in vitro revealed that the drug-induced decrease in airway smooth muscle mass may have been because of reduced migration of myofibroblasts and fibrocytes. —CAC

Sci. Transl. Med. **11**, eaao6451 (2019).

MUCOSAL IMMUNOLOGY

Dietary modulation of T cell immunity

Commensal intestinal bacteria respond to dietary changes by modifying gene expression, leading to shifts in the amounts of bacterial antigens encountered by the intestinal immune system. Wegorzewska *et al.* developed a mouse model system to investigate whether CD4⁺ T cell recognition of antigens of the gut symbiont *Bacteroides thetaiotaomicron* is subject to dietary modulation. T cell receptor–transgenic T cells that recognized a bacterial outer-membrane vesicle protein differentiated into both regulatory and effector T cells, and colitis emerged after selective depletion of the regulatory T cells. Dietary glucose strongly repressed the T cell–detected antigen. Thus, dietary modifications that reduce bacterial expression of immunodominant antigens targeted by T cells could ameliorate some forms of human inflammatory bowel disease. —IRW

Sci. Immunol. **4**, eaau9079 (2019).

REVIEW SUMMARY

MAGNETISM

Two-dimensional magnetic crystals and emergent heterostructure devices

Cheng Gong and Xiang Zhang*

BACKGROUND: The electron can be considered as a tiny magnet, with two opposite poles defining its magnetic field associated with the spin and orbital motion. When such minuscule magnets are collectively aligned as a result of the inherent coupling, ferromagnetism emerges. However, ferromagnetism had long been believed to hardly survive in two-dimensional (2D) systems because of the enhanced thermal fluctuations revealed by the Mermin-Wagner theorem. The recent discovery of 2D magnetic crystals showed that magnetic anisotropy could stabilize the long-range magnetic order by opening up an excitation gap to resist the thermal agitation. Two-dimensional magnetic crystals constitute ideal platforms to experimentally access the fundamental physics of magnetism in reduced dimensions. In contrast to the traditional magnetic thin films, 2D materials largely decouple from the substrates, allow electrical control, are mechanically flexible, and are open to chemical functionalization. These attributes make 2D magnets accessible, engineerable, and integrable into emergent heterostructures for previously unachieved properties and applications such as atomically thin magneto-optical and magnetoelectric devices for ultracompact spintronics, on-chip optical communications, and quantum computing.

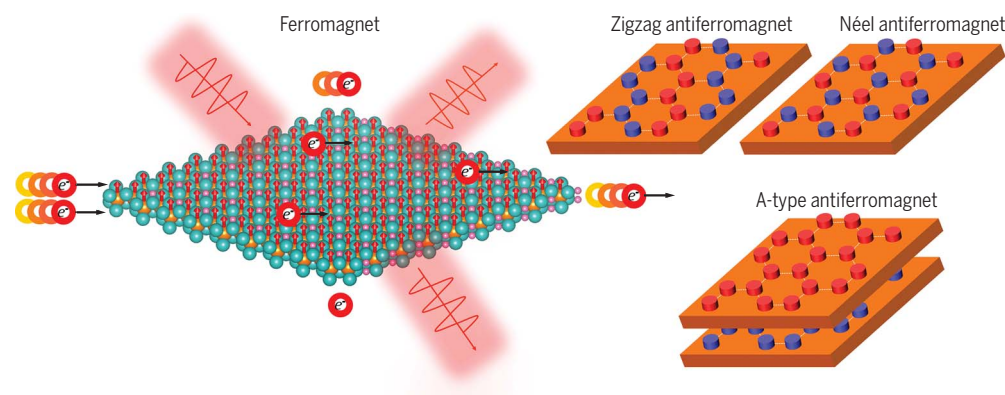
ADVANCES: Magnetism has been explored in 2D materials for more than a decade. Magnetic moments have been created through defect engineering based on vacancies, adatoms, boundaries, and edges; band structure engineering, assisted by density functional theory calculations, has raised possibilities of 2D magnetism in, for instance, gated bilayer graphene and doped GaSe; the proximity effect has been applied to imprint spin polarization in 2D materials from magnetic substrates. However, these prior efforts centered on extrinsically induced magnetic response.

In early 2017, the first observations of long-range magnetic order in pristine 2D crystals were reported in $\text{Cr}_2\text{Ge}_2\text{Te}_6$ and CrI_3 . Both are magnetic insulators, yet with distinct magnetic properties. In contrast, 2D Fe_3GeTe_2 was recently proven to be a magnetic conductor. Itinerant magnets and magnetic insulators possess diverse application perspectives. Molecular beam epitaxial growth of 2D magnets has been reported for Fe_3GeTe_2 , VSe_2 , MnSe_x , and $\text{Cr}_2\text{Ge}_2\text{Te}_6$. The typical Curie temperatures of 2D magnets are much lower than those of their 3D counterparts. However, this does not fundamentally exclude the possibility of high-temperature 2D magnets. Efforts toward this goal have shown promise.

When van der Waals (vdW) magnets contact nonmagnetic materials, time reversal asymmetry could be introduced in the original non-magnets, likely leading to, for example, valley polarization in transition metal dichalcogenides or quantum anomalous Hall states in topological insulators. However, it should be noted that 2D magnets' properties are susceptible to the contacting materials. Stacking vdW magnets with dissimilar materials could enrich the landscape of emergent phenomena by causing, for example, heterostructure multiferroicity, unconventional superconductivity, and the quantum anomalous Hall effect.

Two-dimensional spintronic and magnonic devices have begun to emerge. Spin-orbit torque has been generated while spin-polarized current is injected from 2D materials (e.g., WTe_2) into magnetic substrates; conversely, a spin wave has been pumped from magnetic substrates into 2D materials for spin-charge conversion. Magnetic tunnel junctions with 2D magnets (e.g., CrI_3) as tunneling barriers exhibit giant tunneling magnetoresistance at low temperatures. New concepts of spin field-effect transistors based on 2D magnets have been reported as well.

OUTLOOK: Most currently available 2D magnets rely on mechanical exfoliation and only work at low temperatures. The wafer-scale synthesis of 2D magnets that operate above room temperature is a prerequisite for the development of practical applications. In the longer run, the monolithic integration of such 2D magnets with other functional materials is crucial for practical scalability. Spintronic devices require efficient electrical modulation of 2D magnets, long-distance transport of spins or spin waves, and efficient tunneling and injection of spins at various junctions. The practical development of low-power spintronic devices needs to be compatible with the existing complementary metal-oxide semiconductor technology (e.g., impedance match and affordable power supply). Furthermore, the exotic spin textures, quantum phases, and quasiparticles in 2D magnetic crystals and hetero-interfaces could lead to new ways of computation and communication. We envision that successive breakthroughs in 2D magnets could usher in a new era of information technologies with exciting applications in computing, sensing, and data storage. ■



Two-dimensional magnetic crystals: The atomically thin crystalline hosts of magneto-optic and magnetoelectric effects. 2D magnetic crystals, including 2D ferromagnets (left) and 2D antiferromagnets with diverse intra- and interplane magnetic configurations (right), can exhibit a plethora of magneto-optic and magnetoelectric effects. The red and blue protrusions in the atomic Lego depict the opposite local spins in magnetic layers.

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REVIEW

MAGNETISM

Two-dimensional magnetic crystals and emergent heterostructure devices

Cheng Gong¹ and Xiang Zhang^{1,2*}

Magnetism, originating from the moving charges and spin of elementary particles, has revolutionized important technologies such as data storage and biomedical imaging, and continues to bring forth new phenomena in emergent materials and reduced dimensions. The recently discovered two-dimensional (2D) magnetic van der Waals crystals provide ideal platforms for understanding 2D magnetism, the control of which has been fueling opportunities for atomically thin, flexible magneto-optic and magnetoelectric devices (such as magnetoresistive memories and spin field-effect transistors). The seamless integration of 2D magnets with dissimilar electronic and photonic materials opens up exciting possibilities for unprecedented properties and functionalities. We review the progress in this area and identify the possible directions for device applications, which may lead to advances in spintronics, sensors, and computing.

For centuries, humans had been puzzled about the magic attraction of lodestones to iron, and perhaps even more about the fascinating ability of birds, fish, and insects to navigate between destinations of thousands of miles apart. In early times before the development of electromagnetism and quantum mechanics, it was hard to imagine that these intriguing phenomena may share a common magnetic origin. Magnetism is fundamentally rooted in the moving charges and spin of elementary particles; hence, it is as ubiquitous as the electron itself. It has found broad applications in living organisms as well as in energy harvesting, data storage, and medical diagnosis. When the infinitesimal “electron magnets” are spontaneously aligned, the magnetic order constitutes a fundamental phase of matter, giving rise to a host of functional devices including electric generators and motors, magnetoresistive memories, and optical isolators. The ability to knit such magnetic order in atomically thin flatlands would foster vast opportunities for integrated, flexible, and biocompatible devices; however, such two-dimensional (2D) magnets are not easily attainable because of a fundamental hindrance.

Understanding the fundamental difference between 2D and 3D magnetism is instructive. The driving force underlying the ordering of electrons’ spin magnetic moments in an (anti-)ferromagnet is the “exchange interaction,” which was initially dubbed as a “molecular field” by Pierre Weiss in 1907 (1) and was understood to be of quantum mechanical origin in 1926 (2, 3) (Fig. 1A). The effect is a coulombic interaction under the Pauli exclusion principle relating to the electrons’ anti-

symmetric wave function. The exchange interaction has been used to estimate the Curie temperatures of 3D ferromagnets, based on the argument that the short-range exchange interaction needs to be overcome by thermal energy to randomize the magnetic moments. Nonetheless, the mean-field picture suitable for 3D systems does not work for the length scale of 2D systems, in which the dimensionality effect comes into play (4). Magnon (i.e., quanta of spin wave) dispersion in 2D systems is reduced with respect to that in the 3D counterparts, corresponding to an abrupt onset of magnon density of states (DOS) in 2D systems and thus an ease of thermal agitations (5, 6). For 2D systems without magnetic anisotropy (Fig. 1B), the spin wave excitation gap diminishes (Fig. 1C). Together with the diverging Bose-Einstein statistics of magnons at zero energy, any nonzero temperatures cause massive magnon excitations and the spin ordering to collapse, as predicted by Mermin and Wagner (4). However, for 2D systems with a uniaxial magnetic anisotropy, a magnon excitation gap opens up and resists the thermal agitations (Fig. 1, D and E), which then lifts the Mermin-Wagner restriction and results in finite Curie temperatures. Meanwhile, the exchange interaction together with the dimensionality dictates the magnon band width and profiles (6). Therefore, the synergy of these factors, as well as the inter(quasi-)particle scattering, which potentially renormalizes the magnon spectrum, determines the upper bound temperature (i.e., Curie temperature) below which a 2D ferromagnet can be found.

The past a few decades have witnessed the role of epitaxial thin films and superlattices as testing grounds for the experimental exploration of 2D magnetic properties and spin entity (i.e., spin-polarized electrons or spin waves) propagations. Seminal phenomena such as giant magnetoresistance (7, 8), the dimensionality ef-

fect (9–11), and oscillating exchange coupling (12) were discovered. But it has been a long-standing challenge to access the intrinsic magnetic properties of ultrathin films as a pure quantum confinement effect of their 3D counterparts; these traditional thin films suffer from various perturbations such as interfacial hybridization, electronic redistribution, reduced coordination with band narrowing, atomic interdiffusion, strain, crystalline reconstruction, finite-size islands (typically tens of nanometers), and irregular shapes (13, 14). Therefore, the properties of such ultrathin films are difficult to precisely control and replicate.

In stark contrast, the recently discovered 2D magnetic atomic crystals (6, 15) provide unique opportunities for both fundamental physics and technological advances. Such magnetically ordered atomic crystals enable unprecedented experimental access to the ground states, fundamental excitations, and magnon dynamics of single-crystalline 2D magnets. Because such 2D magnetic crystals are susceptible to a long list of external stimuli including mechanical deformation, electrostatic doping, light incidence, chemical decoration, and dielectric environment, there is enormous room to engineer 2D magnets for desired properties; the sensitive responses of 2D magnets allow the development of miniaturized, lightweight, flexible, and biocompatible devices based on magnetoresistive, magnetoelectric, magnetostrictive, magneto-optical, and magnetobiological effects. Furthermore, the past decade has witnessed the increasingly skillful handling of individual 2D layers, which could facilitate the unprecedented fabrication of multilayer “designer magnets”; a notable outcome could be the giant cross-layer tunneling magnetoresistance by designing the interlayer magnetic coupling. In heterostructures with electronic and photonic materials, the seamless integration and intricate interplay of distinct physical properties could give rise to emergent interfacial phenomena such as heterostructure multiferroicity, unconventional superconductivity, and the quantum anomalous Hall effect. It is reasonable to envision that a vast range of previously unachieved properties will be discovered in 2D magnetic crystals, derivatives, and heterointerfaces, which could be transformed into a host of applications such as low-power spintronics, on-chip optical communications, and quantum computing.

Induced magnetic response in nonmagnetic 2D materials

Since the advent of graphene, attempts to create ferromagnetism in nonmagnetic 2D materials have continued apace. One of the mainstream strategies is through introducing vacancies or adding adatoms such as hydrogen and fluorine (16–22) (Fig. 2, A and B). Such defect engineering produces local magnetic moments from unpaired electrons, which, for example, could be further correlated through conduction electrons in graphene (itinerant π -magnetism). However, attempts to order these moments in a “long-range”

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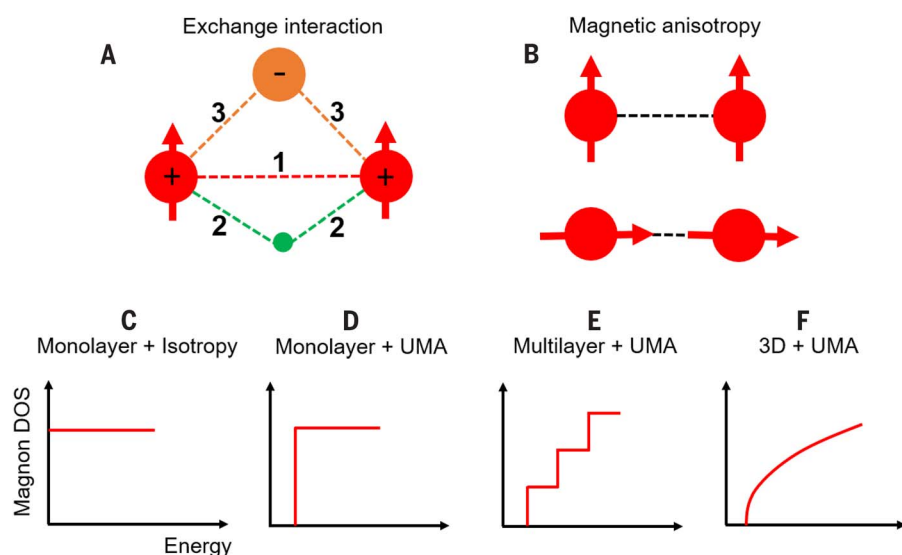


Fig. 1. Fundamental physical parameters and spin wave excitations in ferromagnets of different dimensionalities.

(A and B) In a collinear magnet, exchange interaction and magnetic anisotropy are fundamental parameters. Exchange interaction arises from electrons' antisymmetric wave function and is governed by coulombic interaction under the Pauli exclusion principle. Exchange interaction between spins can be directly established (red dashed line 1) or indirectly mediated by conduction electrons (green ball with dashed lines labeled 2) or intermediate anions (orange ball with dashed lines labeled 3) such as O^{2-} . While spins are aligned, there is usually a preferred orientation, which means magnetic anisotropy. Magnetic anisotropy has a variety of sources such as magnetocrystalline anisotropy, shape anisotropy, and stress anisotropy. (C to F) In a 2D isotropic Heisenberg ferromagnet, there will be massive excitations of magnons at nonzero temperatures because of the absence of a spin wave excitation gap, the abrupt onset of magnon density of states (DOS), and the diverging Bose-Einstein statistics at zero energy; the result is collapse of long-range magnetic order. The presence of uniaxial magnetic anisotropy (UMA) opens up the spin wave excitation gap to resist the thermal agitations of magnons, leading to the finite Curie temperature. As the system evolves from 2D to 3D, the magnon DOS spectrum changes from a step function to a gradually increasing function with zero DOS at the threshold of excitation. Therefore, in 3D systems, UMA (related to the spin wave excitation gap) is not a prerequisite for the presence of finite-temperature long-range magnetic order.

pattern posed overwhelming challenges in material preparation. One report (23) disagreed with the feasibility of these approaches to realizing long-range ferromagnetic order, because the authors only observed the paramagnetic response in graphene with fluorine adatoms or vacancies at liquid helium temperatures, and observed a maximum response of about 1 magnetic moment per 1000 atoms in all tested samples. Despite a number of prior reports on the observation of ferromagnetism in originally nonmagnetic 2D materials, no consensus has been reached (24, 25). The primary debates center on the source of the detected magnetic signals: A chunk of material in a superconducting quantum interference device (SQUID) measurement chamber is typically hundreds of micrometers thick, approximately six orders of magnitude thicker than an atomic sheet. Hence, true signals from the 2D sheet may be overshadowed by ferromagnetic impurities in substrates, even if the impurity concentrations are as low as 10 ppm.

Flat band ferromagnetism has been proposed to be realized via extended defects such as zigzag edges of graphene nanoribbons or grain bound-

daries of 2D materials (26–28). Such defects cause less-dispersed electronic bands that satisfy the huge density of states in a narrow energy scope, leading to the Stoner instability toward a ferromagnetic phase. However, these chemically reactive defects are vulnerable to passivation by foreign species; also, long-range 1D ferromagnetic order cannot exist in theory (26). Although the strict “long-range” (i.e., infinitely long) ferromagnetic order is not allowed in 1D systems, it is possible to make magnetically ordered chains with finite length and width, so that the finite-size ferromagnets (29–31) behave as spin blocks of superparamagnets with reasonably long spin flipping time, which can provide a practical path toward nanoscale spintronic devices (26, 32, 33).

Band structure engineering may represent a vital route to create 2D ferromagnetism from originally nonmagnetic 2D materials, without the assistance of structural imperfections. For instance, such band ferromagnetism was predicted to exist in electrically biased bilayer graphene (34, 35) (Fig. 2F) and doped GaSe (36, 37). In bilayer graphene biased by electric fields perpendicular to the basal plane, the different electrostatic potentials experienced by the two layers

induce a gap opening and a Mexican-hat dispersion at low energy. Such Mexican-hat bands are often concomitant with the itinerant ferromagnetism. Nonetheless, the anticipated ferromagnetic phase has not been experimentally observed thus far, likely because of the limited total magnetic moments or low Curie temperatures. The predicted magnetism in doped GaSe is based on the similar strategy of Mexican-hat band dispersion. However, *ab initio* calculations show the necessity of hole-doped GaSe up to 10^{13} to 10^{14} carriers/cm² to reach the van Hove singularity. Given the large quasiparticle band gap, 3.7 eV (36), it would be challenging to realize this high level of hole doping by conventional electrostatic schemes. Similar spontaneous valley polarization was observed in 2D electronic systems, such as the inversion layer in silicon in the 1970s (38). More recently, giant paramagnetism in monolayer MoS₂ was reported (39), with an unconfirmed yet possible spontaneous ferromagnetism proposed.

The magnetic proximity effect is a scheme to make nonmagnetic 2D materials magnetic by borrowing properties from adjacent magnetic materials. A graphene sheet, which was transferred on yttrium ion garnet (YIG) (40) (Fig. 2D) or upon which EuS was deposited (41) (Fig. 2E), exhibits an anomalous Hall effect. The sole evidence from an anomalous Hall signal does not suffice to conclude that a ferromagnetic phase in 2D materials exists, because other effects such as spin-dependent interfacial scattering or ferromagnetic impurities may result in similar observations (42). However, the quantification of the 14-T interfacial exchange field (41) and the observation of the quantum Hall effect at a much lower external magnetic field shed light on the presence of interfacial exchange fields. More direct evidence of the interfacial exchange field was subsequently obtained on the basis of spin current transport in lateral graphene spin valves on YIG (43, 44). Interestingly, a spin dephasing mechanism due to the temporal and spatial fluctuation of interfacial exchange fields was revealed (44), which highlights from a new angle the critical role of interfacial quality in the spintronic transport properties of proximity systems.

Magnetism in pristine 2D materials

The first two reported 2D magnetic atomic crystals are chromium compounds: Cr₂Ge₂Te₆ (6) (Fig. 3, A to C) and CrI₃ (15) (Fig. 3, D and E). Cr₂Ge₂Te₆ is a 2D Heisenberg ferromagnet with small magnetic anisotropy (i.e., collectively aligned spin moments can be oriented toward all directions with small energy difference), whereas CrI₃ is probably a 2D Ising A-type antiferromagnet (i.e., spin moments oriented normal to the basal plane, intralayer ferromagnetism, and interlayer antiferromagnetism).

The slight distortion of the Cr-Te₆ octahedral cage, together with spin-orbit coupling on Cr ions, leads to a small out-of-plane magnetocrystalline anisotropy in Cr₂Ge₂Te₆. The nearly isotropic Heisenberg 2D ferromagnet mimics the ideal Mermin-Wagner condition, on the basis of which the external magnetic field has an

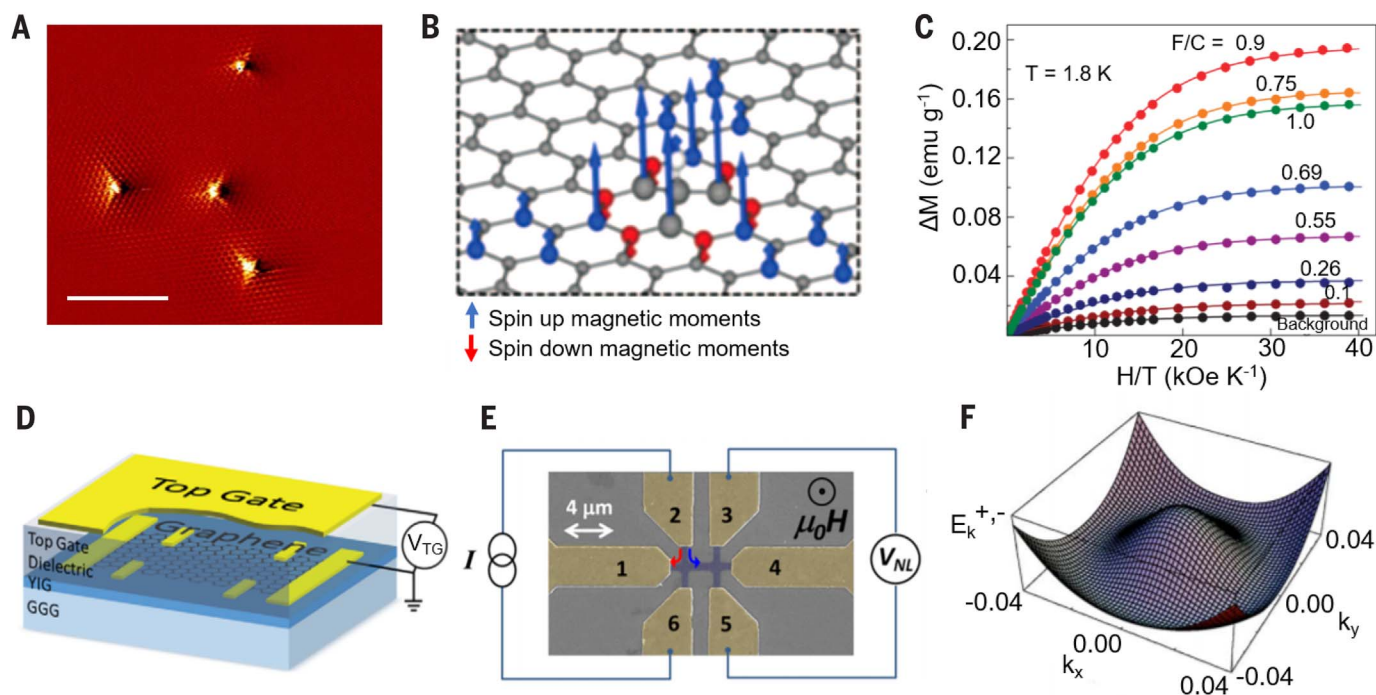


Fig. 2. Schemes to induce magnetism in nonmagnetic 2D materials.

Point defects such as vacancies and adatoms in 2D materials are accompanied by defect states and local magnetic moments. (A) STM topography of graphene with carbon vacancies induced by Ar⁺ ion irradiation (17). Scale bar, 5 nm. (B) Schematic of local magnetic moments in graphene decorated by an individual hydrogen adatom (small white ball at center) (18). The same spin-polarized state extends a few nanometers in carbon sites of the same sublattice, but the opposite spin-polarized state occupies the other carbon sublattice. (C) Magnetization versus magnetic field parallel to the fluorinated graphene planes (23). Dots are experimental data; solid

lines are fitting curves based on Brillouin function. No trace of ferromagnetism was found in both fluorinated graphene and defective graphene with vacancies at liquid helium temperatures. (D) Schematic of a graphene field-effect transistor fabricated on YIG, a magnetic insulator (40). (E) Schematic of a graphene field-effect transistor covered by a deposited thin film of EuS, a magnetic insulator (41). Nonmagnetic 2D materials can be made magnetic by physically contacting magnetic materials through proximity effect. (F) Calculated Mexican-hat band dispersion in electrically biased Bernal stacked bilayer graphene (34). The diverging electronic DOS at the edge of the Mexican hat potentially results in ferromagnetic Stoner instability.

unprecedented effect on the observed transition temperatures of 2D Cr₂Ge₂Te₆. Despite possessing van der Waals (vdW) spacing, interlayer magnetic coupling is appreciable, as evidenced by the strong dimensionality effect in transition temperatures of Cr₂Ge₂Te₆ of different thickness. An isostructural compound, Cr₂Si₂Te₆ (45), has a larger easy-axis magnetic anisotropy and a lower bulk ferromagnetic phase transition temperature T_C at 33 K. The Curie temperature of the hypothetical isostructure Cr₂Sn₂Te₆ is theoretically predicted to be higher (46), but the successful synthesis of this crystal has not yet been reported.

The sizable magnetic anisotropy in CrI₃ was suggested to arise from the exchange anisotropy due to the spin-orbit interaction of iodine species that mediate the superexchange between Cr ions (47). Graphite-encapsulated few-layer CrI₃ shows interesting layer-contrasting magnetic properties and was suggested to be an A-type antiferromagnet. Further investigations are needed to identify the origin of the exotic properties of 2D CrI₃ that are contrary to those of its bulk counterpart [bulk CrI₃ is a ferromagnet (48)], although the altered interlayer registry in few-layer CrI₃ was invoked as a tentative explanation (49–52). Possible extrinsic causes relate to graphite cap-

ping, partial degradation (CrI₃ is extremely unstable to the moisture and light) (53), and unintentional doping and strain (especially for cases where CrI₃ samples were exfoliated on polymer and later transferred). The isostructural magnet CrBr₃ in bulk is an easy-axis ferromagnet with $T_C = 33$ K, and CrCl₃ in bulk is an easy-plane A-type antiferromagnet. Magnetic anisotropies and phase transition temperatures of bulk CrCl_{(3-x)Br_x} alloys are tunable by changing stoichiometries (54).

Fe₃GeTe₂ is a metallic ferromagnet (55–60) (Fig. 3, F and G). In each layer, three of the quintuple sublayers are iron; the top and bottom sublayers are equivalent and the central one differs. The crystallographic environments of the iron atoms are asymmetric along and normal to the basal plane, leading to the sizable magnetocrystalline anisotropy. The tunable Curie temperatures and coercivities can be realized by varying the iron concentrations. Interestingly, in this 3d electronic system, the coexistence of itinerant ferromagnetism and Kondo lattice was evidenced (61), suggesting the presence of heavy fermions and periodically seated local moments. This constitutes an intriguing 2D material platform, in which itinerant electrons and local magnetic moments coexist and interplay, possibly leading to a plethora of emergent phases and phenomena.

Furthermore, for bulk Fe₃GeTe₂, evidence from magnetization characterization, electrical transport (62–64), scanning tunneling microscopy (STM) (65), and magnetic force microscopy (63) points to a different magnetic configuration emerging at even lower temperatures (~50 K lower than the Curie temperature of 220 to 230 K). The stripe domain phase in bulk Fe₃GeTe₂ observed by photoemission electron microscopy (66) indicates the quasi-2D magnetic behaviors, and the thickness-dependent magnetic hysteresis (63, 64) reveals valuable hints that long-range dipolar interaction may play an important role when layers are stacked up to tens of nanometers.

In contrast to ferromagnets, antiferromagnets find limited applications—for example, to stabilize the “fixed layer” in spin valves and magnetic tunnel junctions. Despite the notorious difficulty in using antiferromagnets due to their net vanishing magnetization, antiferromagnets hold promise for high-speed, low-power spintronics because they have magnetic resonance frequencies in the terahertz regime, null stray field for vanishing cross-talk between adjacent bits, and robustness against the external magnetic field perturbation (67, 68). Furthermore, antiferromagnets are much more abundant than ferromagnets.

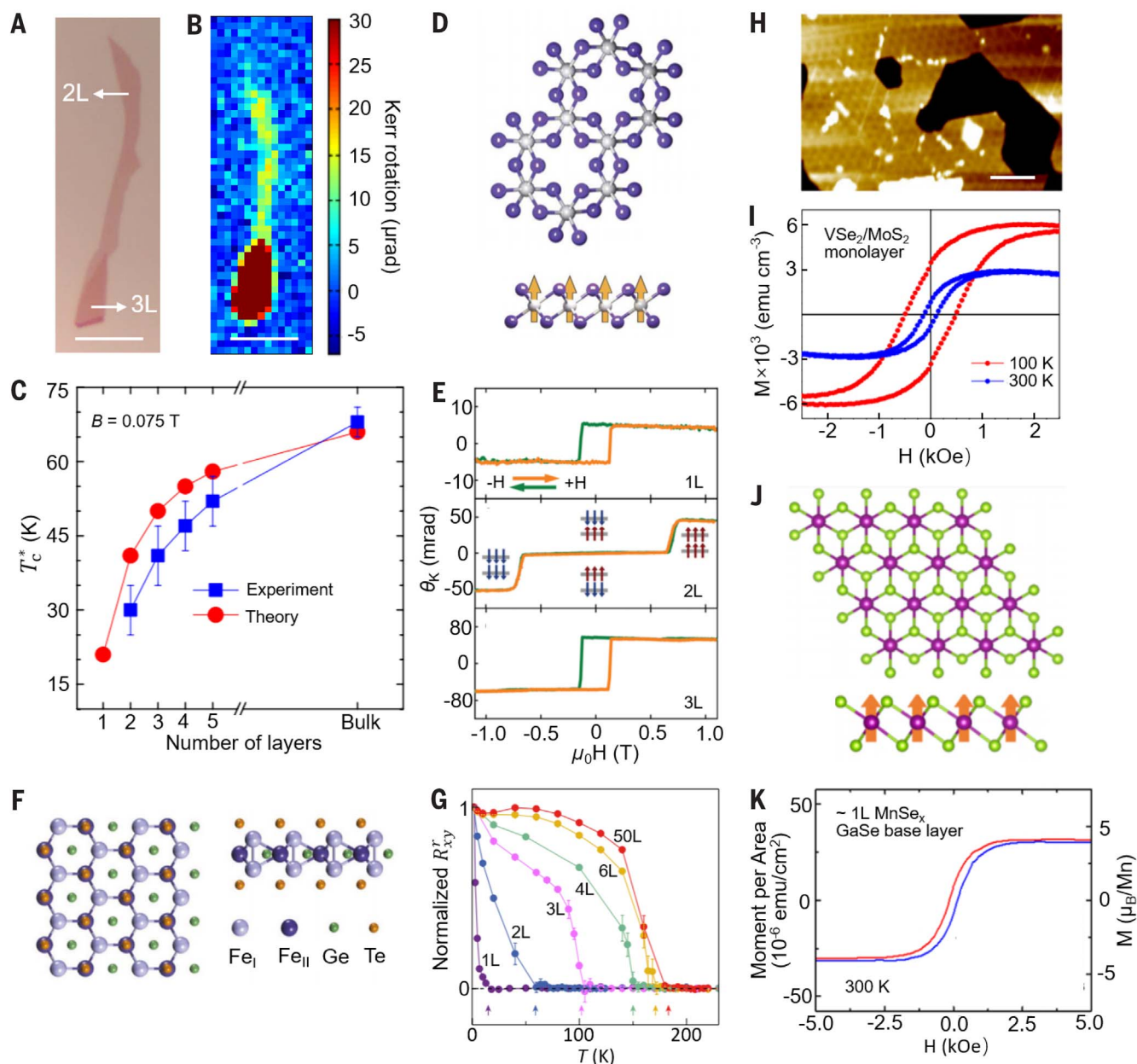


Fig. 3. Representative 2D magnetic crystals. (A to C) Optical image, Kerr image, and dimensionality effect of few-layer $\text{Cr}_2\text{Ge}_2\text{Te}_6$ exfoliated on SiO_2/Si (6). Scale bars, 10 μm . (D and E) Atomic structure of CrI_3 and thickness-dependent Kerr signal hysteresis loop of graphite-sandwiched 2D CrI_3 (15). In (D), orange arrows represent the ferromagnetically coupled spin magnetic moments. In (E), red and blue vertical arrows represent spin-up and spin-down magnetic moments, respectively. (F and G) Atomic structure of Fe_3GeTe_2 and thickness-dependent normalized remanent anomalous Hall resistance of 2D Fe_3GeTe_2 on Al_2O_3 thin film, which was prepared by thermally evaporating Al in oxygen

pressure of 10^{-4} mbar on Fe_3GeTe_2 bulk crystal, followed by multiple steps of transfer and exfoliation (62). (H and I) STM image of sub-monolayer VSe_2 grown on HOPG by MBE and magnetic hysteresis of mostly monolayer VSe_2 grown on MoS_2 by MBE (75). Scale bar, 20 nm. (J and K) Atomic structure of vdW MnSe_2 and out-of-plane magnetic hysteresis of MnSe_x , one monolayer on average, grown on GaSe by MBE (76). In (J), orange arrows represent the ferromagnetically coupled spin magnetic moments. In (K), red and blue curves represent the half of the hysteresis loop while magnetic field is swept from positive to negative and from negative to positive, respectively.

Antiferromagnetic vdW materials are diverse in magnetic configurations such as the Ising antiferromagnets FePS_3 and FePSe_3 (69–72), the Heisenberg antiferromagnets MnPS_3 and MnPSe_3 (72, 73), and the XY-type antiferromagnet NiPS_3 (73). Raman spectroscopy could provide nondestructive techniques to indirectly probe the magnetic phase transitions for some anti-

ferromagnets. For example, FePS_3 has a Raman feature peak whose intensity has a sharp upturn when samples are cooled under Néel temperature (74). Methods that can effectively characterize antiferromagnetic order and magnetic excitations in atomically thin micrometer-size antiferromagnetic samples would be useful. Reading information from 2D antiferromagnets

would pave the way to taking full advantage of the vdW antiferromagnets for ultrafast low-power spintronics.

An important consideration for the practical use of 2D magnets is the magnetic phase transition temperature. Substantial work has been devoted to enhancing the Curie temperatures of 2D ferromagnets beyond room temperature, with

a few claims of success thus far. For this ambitious goal, there appears to be no fundamental prohibition as long as the ferromagnetic exchange interaction and uniaxial magnetic anisotropy are strong enough. Yet the practical realization is not that easy. Room-temperature ferromagnetism was reported in single-layer 1T-VSe₂ synthesized on both highly oriented pyrolytic graphite (HOPG) and MoS₂ by molecular beam epitaxy (MBE) (75) (Fig. 3, H and I). Two puzzling points remain for this work: the unusually high magnetic moments (8000 emu/cm³) in single-layer VSe₂ on MoS₂, and the observed easy-plane anisotropy, which can hardly sustain the long-range ferromagnetic order at room temperature unless a strong uniaxial anisotropy is present within the basal plane. In another contemporary study, the vdW phase of single-layer MnSe_x was synthesized by MBE (76) (Fig. 3, J and K). Interestingly, such a vdW phase for MnSe_x only exists in ultrathin layers, but the bulk counterparts prefer rock-salt NaCl or the hexagonal NiAs phase, both of which are antiferromagnetic (77). This observation highlights that 2D materials could exhibit novel structural forms that are not present in bulk, and thus possess unusual physical properties. For MBE samples, the possibly complicated interfaces formed during sample synthesis (e.g., by atomic diffusion, reaction, and alloying) should be carefully examined as the probable causes of the observed magnetic signals. A recent work (78) pointed out the magnetic element in a hot cell as a source of magnetic impurities in MBE synthesis.

In theory, there is not a fundamental restriction to 2D long-range magnetic order at high temperatures, although enhanced thermal fluctuations are always a hindrance. The rule of thumb in designing high-temperature 2D ferromagnets is to strengthen the exchange interaction and uniaxial magnetic anisotropy. Given the extensive prior experience in creating magnetic moments and enhancing spin-orbit coupling in graphene and other nonmagnetic 2D materials, it is safe to envision that thermally robust 2D ferromagnets will be discovered in ever more engineered 2D material systems.

Magnetism as a result of many-body interaction suggests electron correlation effects. Spin-charge coupling has been evidenced in NiPS₃ (79), an easy-plane antiferromagnet, showing the presence of electron correlations. Antiferromagnetism and strong electron correlations are reminiscent of unconventional superconductors. Interestingly, a recent experiment showed that FePSe₃, a vdW antiferromagnet, indeed evolves to be superconducting under hydrostatic pressure above 9 GPa (80). For ferromagnetic Cr₂Ge₂Te₆, the sharp upturn of electrical resistivity when temperature is lowered to a certain point above *T*_C suggests the spin-charge coupling (81). Strong electron correlations are implied by the enhanced electronic specific heat of Fe₃GeTe₂ with respect to that of nonmagnetic isostructural Ni₃GeTe₂, and are also supported by the comparison between experimental and calculated Sommerfeld coefficients in Fe₃GeTe₂ (57). Therefore, on the basis of all of this evidence, electron correla-

tions appear generally present in 2D magnetic crystals. Such electron correlations enrich the complex phase diagrams, allow the electrical tuning and compositional engineering to switch between multiple phases, and make heterostructures involving 2D magnets fertile grounds for emergent interfacial phenomena.

Control of 2D magnetism

Electric control of low-dimensional magnetic systems (82, 83), through either an electric field or electrostatic doping, changes the electron population, orbital occupation, and possibly electrochemical reactions (84), leading to the modification of exchange parameters and magnetic anisotropies and thus the resultant magnetic properties. Electrical control of recently emerged 2D magnets has been achieved. In bilayer antiferromagnetic CrI₃, both electric fields and electrostatic doping can affect the spin-flipping magnetic field (85–87). One remarkable phenomenon is the almost complete conversion of the few-layer graphene-encapsulated bilayer CrI₃ from interlayer antiferromagnetism to ferromagnetism by electrostatic doping (87). The Curie temperature of single-layer CrI₃ was modulated between 40 K and 50 K by changing doping levels. Electrical control of few-layer Cr₂Ge₂Te₆ via ionic liquid gating shows the modulation of coercivity and saturation field (88). In contrast to magnetic insulators, the itinerant nature of ferromagnetism in Fe₃GeTe₂, given the magnetism is mediated through conductive electrons, possibly allows more effective tuning of Curie temperatures by controlling the carrier concentrations within. The hysteresis loop of few-layer Fe₃GeTe₂ was recently reported in anomalous Hall effect measurements at room temperature (62), while the 2D magnet was electrostatically doped via an ionic liquid. Scanning microscopy may provide further information on whether a long-range ferromagnetic order or local magnetic islands are formed, considering that both structural distortion and electrochemical reaction possibly occur when 2D materials meet ionic liquids. Also, the conversion between antiferromagnetism and ferromagnetism was predicted to occur in both electron- and hole-doped MnPSe₃ above 3×10^{13} to 4×10^{13} carriers/cm² (89). Such antiferromagnetism-ferromagnetism conversion may prompt a new means of magnetization switching for logics and memories.

Magnetoelectric multiferroics could enhance the efficiency of electric control of magnetism because of the inherent coupling of magnetic and electric orders. Magnetoelectric multiferroics that simultaneously possess ferromagnetism and ferroelectricity hold great promise in next-generation spintronics and microwave magnetoelectric applications. However, in the 2D regime, each type of ferroic order has its own hindrance, such as the enhanced thermal fluctuations for 2D ferromagnetism and the strong depolarization fields for 2D ferroelectricity. Despite the recent success in realizing 2D ferromagnetism and 2D ferroelectricity (90–94) in different 2D materials, the simultaneous realization of both orders in

one 2D material has not been reached. The theoretically predicted 2D multiferroics are primarily of ferromagnetism and ferroelasticity [e.g., monolayer group IV mono-oxide α -SnO (95)] and of ferroelectricity and ferroelasticity [e.g., monolayer group IV monochalcogenides such as GeS, GeSe, SnS, and SnSe (96) and monolayer ternary compounds such as GaTeCl (97)]. At the time of writing, very few theoretical predictions have been made on 2D magnetoelectric multiferroics (98, 99). An unusual 2D magnetoelectric multiferroic was predicted on the basis of hyperferroelectric CrN (98). The scarcity of single-phase 2D magnetoelectric multiferroics relates to the longstanding challenge in multiferroics physics: Ferromagnetism prefers partially filled *d*-orbitals, whereas ferroelectricity prefers empty *d*-orbitals for displacive movement of ions.

Another knob for the effective control of magnetism is pressure or strain. Magnetic properties critically hinge on materials' structural parameters. Thus, spin-lattice coupling and magnetostrictions are common phenomena in magnetic materials. Spin-lattice coupling has been experimentally observed or theoretically predicted in layered magnets such as Cr₂Si₂Te₆ (45), Cr₂Ge₂Te₆ (100), and Fe₃GeTe₂ (58). While being cooled into ferromagnetic phase, both the in-plane lattice constant and the interlayer spacing of Cr₂Si₂Te₆ undergo the increase (45). For Cr₂Ge₂Te₆, frequencies of a few phonons exhibit upturns while being cooled into a ferromagnetic phase, highlighting the spin-phonon coupling (100). A hydrostatic pressure of 1 GPa can reduce the Curie temperature of the bulk Cr₂Ge₂Te₆ by ~9% (101). Furthermore, hydrostatic pressures above 1 GPa can reorient the spins of the bulk Cr₂Ge₂Te₆ from out-of-plane to in-plane (102). As discussed above, pressurized FePSe₃ even underwent a transformation from antiferromagnetic insulator to superconductor. These findings showcase the effectiveness of pressure or strain in engineering 2D magnets.

Heterostructures and interfacial engineering

High spin and valley polarizations in 2D materials provide tantalizing opportunities for efficient spintronics and valleytronics. Placing 2D electronic or valleytronic materials on magnetic insulators offers an effective methodology to spin-polarize and/or valley-polarize 2D materials. Especially if such magnetic insulators are also vdW materials, the seamless integration and interplay of vdW heterostructures could benefit the interfacial exchange interaction due to atomically sharp interfacial registry. Various material systems in which the proximity effect has been used to spin- or valley-polarize 2D materials include graphene on YIG (40), EuS on graphene (41), WSe₂ on EuS (103), and WSe₂ on CrI₃ (104).

Time-reversal symmetry breaking in 3D topological insulators can induce a gap opening in the 2D Dirac surface states, possibly giving rise to quantum anomalous Hall states on 1D edges. The conventional way to realize the quantum

anomalous Hall effect (QAHE) is through magnetic dopants, such as Cr-doped Bi_2Se_3 . However, magnetic impurities scatter the electrons' traveling and suppress the temperature limit for the realization of QAHE. Given the atomically flat vdW ferromagnet, the sharp interfacial registry may effectively magnetize the surfaces of topological insulators without incurring structural perturbations. Such endeavors have been attempted using metal-organic chemical vapor deposition of Bi_2Te_3 on $\text{Cr}_2\text{Ge}_2\text{Te}_6$ (105) and MBE growth of $\text{Cr}_2\text{Ge}_2\text{Te}_6$ on $(\text{Bi,Sb})_2\text{Te}_3$ (106). Another surge of interest toward QAHE was triggered by the recent advances in the synthesis of intrinsic magnetic topological insulators such as MnBi_2Te_4 and MnBi_2Se_4 (107–112). These layered materials are not only intrinsically magnetic but also topologically nontrivial. Rather than randomly distributing in the Bi sites substitutionally, Mn self-organizes in the center layer of the septuple layers of, for example, $\text{Mn}_2\text{Bi}_2\text{Te}_4$, which is an A-type antiferromagnetic topological insulator and may host the quantized topological magnetoelectric effect, quantum anomalous Hall state, and intrinsic axion insulator state. Successful synthesis of $\text{Mn}_2\text{Bi}_2\text{Te}_4$ in both thin films and millimeter-size bulk single crystals (108) has been reported.

Placing 2D vdW magnets in contact with other materials not only lends the magnetic properties of 2D magnets to others, but can also modify the 2D magnets via interfacial engineering. A remarkable example is the magnetic phase at the $\text{EuS-Bi}_2\text{Se}_3$ interface persisting beyond room temperature (113). Interfacial engineering represents a vital scheme to tailor 2D magnetic properties: It is based on artificial material systems, which means tremendous freedom relative to the design and synthesis of single-phase materials. Moreover, unlike the modulation of magnetic properties through external stimuli such as electrical field, interface engineering does not require external power sources to maintain the modified properties.

A host of factors make possible the interface engineering of 2D magnets, as summarized in Fig. 4:

1) The interface charge transfer changes the electron concentration and orbital occupation in 2D magnets, leading to the property change.

2) The interface dipole or built-in electric field can modify the electronic structure or crystal field of 2D magnets.

The above two factors were proposed to explain the nonzero remanent magnetization in bilayer CrI_3 sandwiched by few-layer graphene [figure 3a of (87)] and the different magnetic properties between graphite-bilayer CrI_3 -BN and BN-bilayer CrI_3 -BN [figure S4 of (85)]. The interface effects in the resultant 2D magnetic properties were also reflected by the distinct coercivities in different regions of the same monolayer CrI_3 flake sandwiched by graphite [figure 2d of (15)].

3) The interfacial orbital hybridization affects the resultant magnetic property of 2D magnets by impinging on the electronic properties and orbital characters of 2D magnets.

4) The morphology and lattice strain of 2D magnets can be modified when 2D magnets interface with other materials, resulting in the change of 2D magnetic properties.

5) Exchange anisotropy and the superexchange mediated by outermost atoms of 2D magnets are susceptible to the contacted materials.

6) The band structure of a 2D magnet may renormalize when interfacing with a material of a similar lattice constant. For example, graphene band properties can be strongly altered by *h*-BN. Band renormalization of 2D magnets can also be caused by the dielectric screening of adjacent materials.

7) The dielectric environments screen the coulombic interaction (note: in nature, exchange interaction is a coulombic interaction). Such dielectric screening of electron-electron interaction has been well studied for mobility enhancement in transistors (114) and has been shown to effectively weaken the excitonic binding energy in 2D materials [the adjacent single-side bilayer

graphene reduces the excitonic binding energy in MoSe_2 by ~ 100 meV (115)].

8) The spin-orbit coupling proximity can play a role when 2D magnets are in contact with heavy elements, given that the magnetocrystalline anisotropy is intrinsically related to the spin-orbit coupling. For example, graphene's spin-orbit coupling strength was enhanced three orders of magnitude by contacting WS_2 (116).

Device applications: 2D spintronics, magnonics, and spin-orbitronics

The magnetic tunnel junction (MTJ) is a fundamental building block for the state-of-the-art spintronic industry (7, 8, 117–123). One of the obvious advantages in all-vdW MTJs is that the uniform barrier thickness facilitates the all-area tunneling. In contrast, tunneling in nonuniform MTJs preferentially occurs through thinner barrier regions, because the tunneling current is an exponential function of the barrier thickness. Such vdW MTJs have been pursued based on

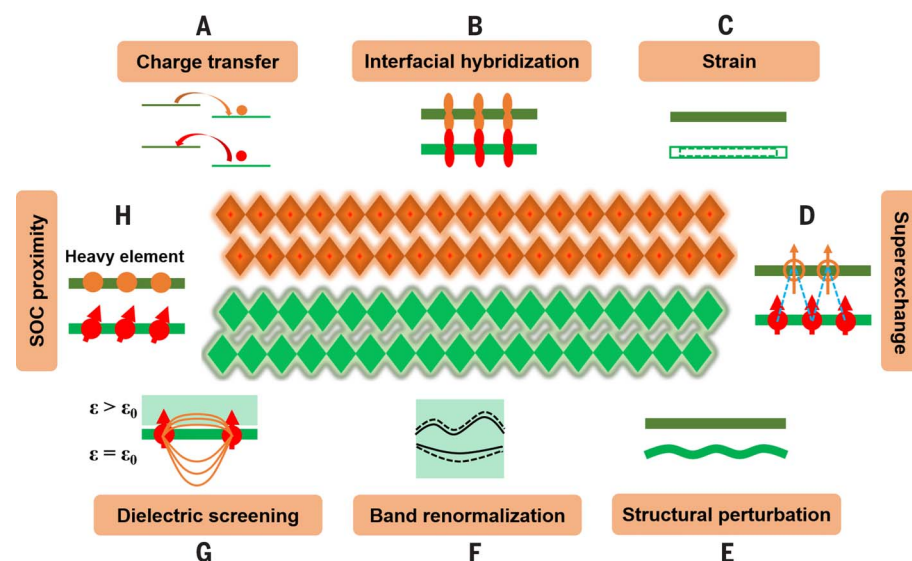


Fig. 4. Interfacial engineering of 2D magnets. Magnetic properties of 2D magnets can be affected by adjacent materials via different mechanisms. The central structure depicts an interface between a 2D magnet (green) and a dissimilar material (orange). (A) Charge transfer and interfacial dipole. The orange and red balls represent electrons and holes, respectively. (B) Interfacial hybridization. The lower green bar represents a 2D magnet; the upper bar is a dissimilar material. The dumbbells represent electronic orbitals of the two materials, overlapping at the interface to hybridize. (C) Strain effect. The lower solid bar represents a stretched 2D magnet in contact with a dissimilar material; the lower dashed bar represents the relaxed 2D magnet without the top contacted material. (D) Additional superexchange interactions. The orange circles with arrows represent the elements in adjacent materials that provide additional channels to mediate the superexchange interaction between magnetic ions in 2D magnets, which are represented by the red solid balls with arrows. (E) Structural perturbation. The wavy green belt represents 2D magnets that are structurally perturbed because of contact with the adjacent materials. (F) Band renormalization. The solid curves represent the electronic, magnonic, or phononic band dispersions of 2D magnets after band renormalization with contact of the adjacent materials; the dashed curves represent the same band dispersions before band renormalization without contacting the adjacent materials. (G) Dielectric screening. Red balls with arrows represent the exchange-coupled electrons in 2D magnets; orange curves depict the electric field lines connecting electrons. The environment with higher dielectric constant ϵ screens the coulombic interaction more. The nature of exchange interaction as a coulombic interaction makes 2D magnets susceptible to the dielectric screening. (H) Spin-orbit coupling (SOC) proximity. By contacting heavy-element materials, the SOC in 2D magnets will be effectively modified, leading to the change of magnetocrystalline anisotropy.

$\text{Fe}_{0.25}\text{TaS}_2\text{-Ta}_2\text{O}_5\text{-Fe}_{0.25}\text{TaS}_2$ (124), graphite- CrI_3 -graphite (49, 125–127), $\text{Fe}_3\text{GeTe}_2\text{-BN-Fe}_3\text{GeTe}_2$ (128), and graphite- CrBr_3 -graphite (129). The Curie temperatures of Fe_xTaS_2 are tunable by Fe's stoichiometry with maximum T_C at ~ 160 K when $x = 0.2$, and Ta_2O_5 is a thin non-vdW native oxide (Fig. 5, A and B). The tunneling magnetoresistance is about 6.5% at 5 K (124). Recently, several research groups reported the very large tunneling magnetoresistance based on graphite- CrI_3 -graphite sandwich structures (Fig. 5, C and D), with maximal magnetoresistance amounting to 19,000% at 2 K (125), 550% at 300 mK (126), 10,000% at 10 K (49), and 1,000,000% at 1.4 K (127). The large variance among these reported tunneling magnetoresistance values relates to such detailed experimental conditions as measurement temperature, thickness of the spacing layer, applied bias, and the detailed interfacial quality. The key of this type of MTJ was deemed to make use of the multiple scattering of tunneling electrons across the alternatively spin-polarized CrI_3 layers. These MTJs were found to be a result of the magnon-mediated tunneling process, in contrast to the conventional phonon- or electron-mediated tunneling (126, 129). Another vdW MTJ, $\text{Fe}_3\text{GeTe}_2\text{-BN-Fe}_3\text{GeTe}_2$, exhibited 160% tunneling magnetoresistance at 4.2 K (128). These proof-of-concept studies show the attractive promise of vdW magnets in high-efficiency spintronics or magnonics.

To be balanced, outstanding challenges remain in these vdW MTJs concerning room temperature, nonvolatility, and low-power switching. For example, the current version of CrI_3 -MTJs works at about liquid helium temperatures, is volatile, and requires large magnetic fields for toggling between distinct states (e.g., 2 T for tetralayers). For practical vdW MTJs, efforts need to be directed toward such important issues as high-temperature 2D magnets, perpendicular anisotropy, large remanence, modest coercivity, and robust exchange bias with antiferromagnets. Nonetheless, despite challenges, it is hoped that the smaller magnetic volume in ultrathin vdW MTJs, better electrical control, and enhanced thermal fluctuations in 2D magnets could allow a lower critical current for spin torque magnetization switching relative to the traditional non-vdW MTJs. This lower critical current is the key long-sought goal for spin-transfer torque magnetoresistive random-access memory (STT-MRAM).

Bilayer structures consisting of 2D and magnetic materials provide intriguing opportunities in magnonics and spin-orbitronics. Spin pumping and spin-orbit torque are reciprocal processes. Magnons can be excited in magnetic substrates coherently by microwave. The excited magnons, without the necessity of conducting electrons, then propagate into the adjacent 2D materials. If the 2D materials have large spin-orbit coupling strengths or large inverse Rashba-Edelstein coefficients, they would be capable of efficiently converting the traveling magnons into charge current and detectable voltage. Proof-of-concept experiments on such spin-charge conversion have been conducted in graphene-YIG (130, 131)

(Fig. 5E), $\text{MoS}_2\text{-Al-Co}$ (132), and $\text{MoS}_2\text{-YIG}$ (133) heterostructures.

In a reverse manner to spin pumping, spin-transfer torque or spin-orbit torque results from an injection of spin current from a 2D material

into a magnetic thin-film substrate. A longitudinal charge current in a 2D material with strong spin-orbit coupling can deflect electrons of transverse spin orientations toward the directions normal to the bilayer interface, resulting in the injection of a

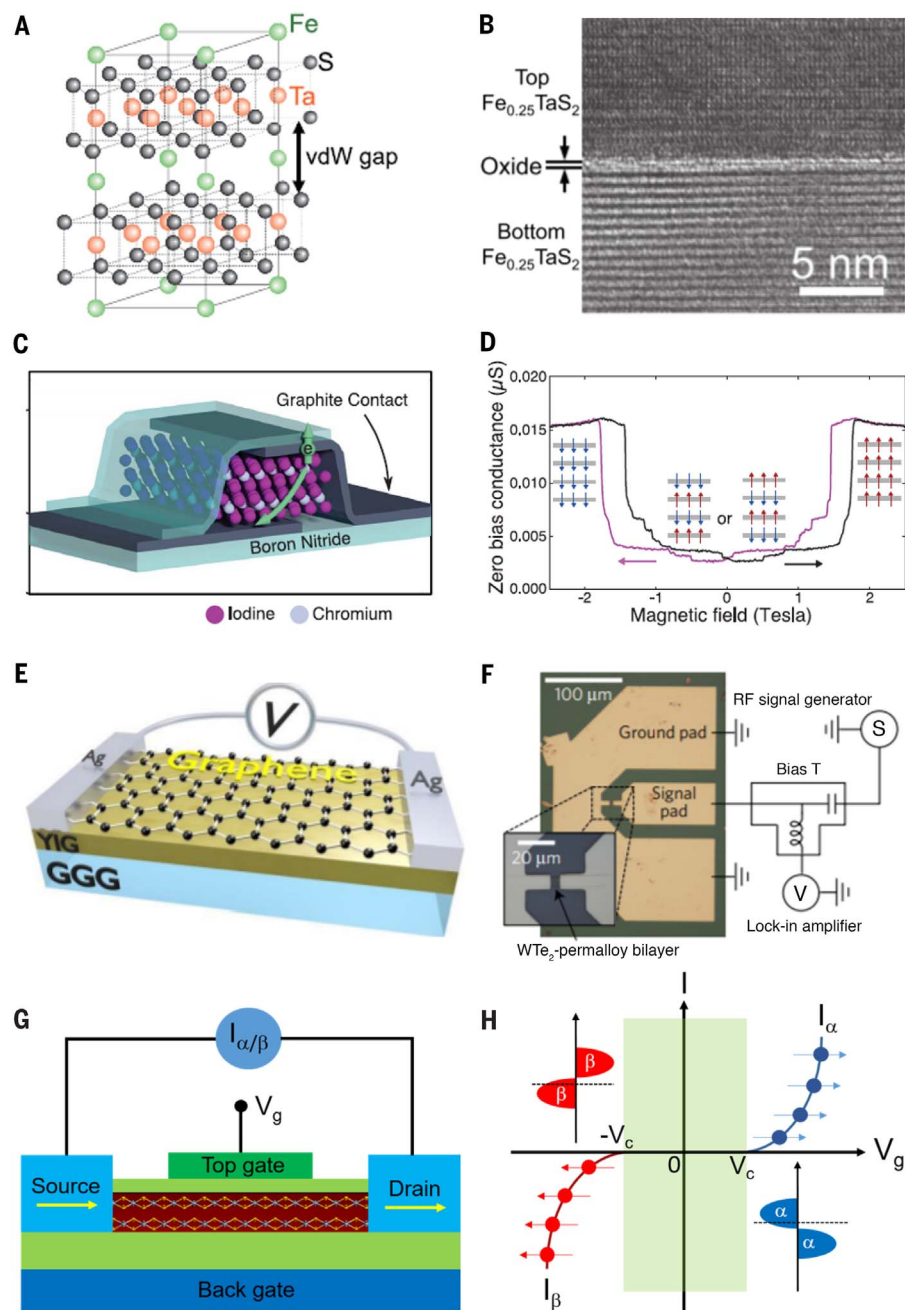


Fig. 5. Spintronic, magnonic, and spin-orbitronic devices based on 2D magnets or magnetic heterostructures. (A and B) MTJ based on $\text{Fe}_{0.25}\text{TaS}_2\text{-Ta}_2\text{O}_5\text{-Fe}_{0.25}\text{TaS}_2$ (124). Iron-intercalated TaS_2 is ferromagnetic, and the surface native oxide was used as an insulating spacing layer. (A) Atomic structure of Fe-intercalated TaS_2 . (B) Cross-section transmission electron microscopy image of the MTJ sandwich structure. (C and D) MTJ based on graphite- CrI_3 -graphite (126). (C) Schematic of MTJ. (D) Magnetic field-dependent tunneling conductance. (E) Schematic of graphene-YIG heterostructure for spin-charge conversion based on spin pumping (130). (F) Schematic of the spin-orbit torque measurement system, for which the core material architecture is WTe_2 -permalloy heterostructure (135). Inset is an optical image of the tested device. (G and H) Schematics of a spin field-effect transistor based on a bilayer A-type antiferromagnet and its predicted electrical properties (140).

Chalcogenides	Cr ₂ Ge ₂ Te ₆ , Cr ₂ Si ₂ Te ₆ , Fe ₃ GeTe ₂ , VSe ₂ [*] , MnSe _x [*]	Fe ₂ P ₂ S ₆ , Fe ₂ P ₂ Se ₆ , Mn ₂ P ₂ S ₆ , Mn ₂ P ₂ Se ₆ , Ni ₂ P ₂ S ₆ , Ni ₂ P ₂ Se ₆ , CuCrP ₂ Se ₆ [*] , AgVP ₂ S ₆ , AgCrP ₂ S ₆ , CrSe ₂ , CrTe ₃ , Ni ₃ Cr ₂ P ₂ S ₉ , MnBi ₂ Te ₄ [*] , MnBi ₂ Se ₄ [*]	CuCrP ₂ S ₆
Halides	CrI ₃ [*] , CrBr ₃ , GdI ₂	CrCl ₃ , FeCl ₂ , FeBr ₂ , FeI ₂ , MnBr ₂ , CoCl ₂ , CoBr ₂ , NiCl ₂ , VCl ₂ , VBr ₂ , VI ₂ , FeCl ₃ , FeBr ₃ , CrOCl, CrOBr, CrSBr, MnCl ₂ [*] , VCl ₃ [*] , VBr ₃ [*]	CuCl ₂ , CuBr ₂ , NiBr ₂ , NiI ₂ , CoI ₂ , MnI ₂
			α-RuCl ₃
Others	VS ₂ , InP ₃ , GaSe, GaS	MnX ₃ (X = F, Cl, Br, I), FeX ₂ (X = Cl, Br, I), MnSSe, TiCl ₃ , VCl ₃	SnO, GeS, GeSe, SnS, SnSe, GaTeCl, CrN, CrBr ₂

Fig. 6. Van der Waals magnets library. Color code: Green, bulk ferromagnetic vdW crystals; orange, bulk antiferromagnets; yellow, bulk multiferroics; gray, theoretically predicted vdW ferromagnets (left), half metals (center), and multiferroics (right), which have not yet been experimentally confirmed; purple, α-RuCl₃ (a proximate Kitaev quantum spin liquid) (144). Notes (asterisks): VSe₂ has been found only in 1T-VSe₂ form in experiments to date (145), although the magnetic properties of 2H-VSe₂ have been widely studied by density functional theory calculations. MnSe_x is ferromagnetic and of vdW structure in MBE-synthesized 2D form but is antiferromagnetic in bulk (which could be either rock-salt or hexagonal structure). CrI₃, although ferromagnetic in bulk, was experimentally suggested to be

an A-type antiferromagnet in the 2D regime. CuCrP₂Se₆ does not host the electric order while being cooled down to 10 K according to experimental data (147), but the calculated ground state of CuCrP₂Se₆ is multiferroic with antiferroelectricity (99). MnBi₂Te₄ and MnBi₂Se₄ may exhibit ferrimagnetic features as a result of uncompensated odd-layer A-type antiferromagnets or surfaces of antiferromagnetic topological insulators. MnCl₂ has a magnetic structure that has not been completely determined, which could be either antiferromagnetic or helimagnetic (142). Bulk VCl₃ and VBr₃ have been inferred to be weak antiferromagnets on the basis of experimental data, although detailed magnetic structures have not been determined; however, monolayer VCl₃ was calculated to be ferromagnetic (146).

spin-polarized current into the magnetic substrate. The injected spin current exerts spin-orbit torques on the magnetic moments of the underlying magnetic substrate, exciting the moments to precess or even switch to the opposite orientation. Spin-orbit torque MRAM, if using 2D materials as the spin-current sources, has the potential toward the atomic thinness. The other advantage of using 2D materials as a spin-current source is that the majority of the spin current would be at the interface rather than being dissipated in bulk, constituting a tantalizing platform for low-power spintronics. Many types of bilayer structures consisting of 2D materials such as MoS₂-permalloy (134), WTe₂-permalloy (135) (Fig. 5F), and (MoS₂ and WSe₂)-CoFeB (136) have been investigated.

Lastly, it is worthwhile to note the family of half metals. To improve spintronics, high spin polarization at the Fermi level of a material is desirable. Half metallicity, a property arising from the metallic nature for electrons with singular spin polarization and insulating for electrons with the opposite spin, holds the potential for 100% spin-polarized conduction electrons. There have been a few theoretical predictions on the possible 2D half metals, including manganese trihalides (137), FeCl₂, FeBr₂, FeI₂ (138), and Janus structure of monolayer MnSSe (139). Half metallicity could also be electrically induced in bilayer A-type antiferromagnets (e.g., in 2H-VSe₂) (140). This electrically induced half metallicity potentially prompts a new type of spin-field-effect transistor in which both switching and reversal of spin polarization can be realized by a single knob: gate voltage, in analogy to the conventional semiconductor transistors (Fig. 5, G and H).

Conclusions and outlook

The recently discovered 2D magnetic crystals provide ideal platforms to study the ground state, fundamental excitations, dynamics, and frustrations of spin ensembles under strong quantum

confinement. Such intrinsic 2D magnetic crystals distinguish themselves from traditional magnetic thin films and nonmagnetic 2D materials, in terms of both properties and application prospects. The interplay of dimensionality, correlation, charge, orbital character, and topology makes 2D magnetic crystals and heterostructures extremely fertile condensed matter systems with a large reservoir of exotic properties. The 2D materials' outstanding feature of being susceptible to a large variety of external stimuli makes the versatile control of 2D magnetism possible by electrical, mechanical, chemical, and optical approaches. Furthermore, because of the unrivaled compatibility for heterostructure constructions, vdW heterostructures present attractive opportunities for designer 2D magnetic, magnetoelectric, and magneto-optical artificial heteromaterials. Those materials that contact 2D magnets will affect the 2D magnetic properties. Novel spintronic, magnonic, and spin-orbitronic devices have started to emerge.

As evident in Fig. 6, a large variety of magnetic vdW materials are available (141–148), most of whose 2D counterparts remain to be studied. The family of 2D magnetic crystals is rapidly growing, magnetic heterostructures consisting of 2D materials are being actively expanded, and new device concepts are being developed. One of the most critical needs concerns the material-level realization of room-temperature 2D ferromagnets (in contrast to sustaining a high Curie temperature with external supplies such as electrical, optical, and mechanical means), which ideally would be air-stable. Furthermore, the wafer-scale synthesis (149) of such crystals has to be accomplished for practical mass production. Advances in both metallic and insulating 2D magnets, both 2D ferromagnets and antiferromagnets, promise various aspects of applications based on their distinct properties.

Fundamental spintronic parameters—including spin Hall angle, spin diffusion length, magnon

damping coefficient, spin injection efficiency, and spin mixing conductance—must be evaluated in 2D magnets and across interfaces. There is a need for in-depth understanding of the relation between spintronic transport metrics and material parameters, along with the corresponding strategies for optimization. Furthermore, exotic spin textures and topologically protected spin configurations (e.g., magnetic skyrmions) in 2D magnets or heterostructures remain open to explore by rationally weighing the material constituents, crystal symmetry, spin-orbit coupling, Rashba effect, and spin (re-)orientations. New quantum phases and quasiparticles could be found, leading to new ways of computation and communication. Such advances in understanding and control of 2D magnetic crystals and emergent heterostructure devices will foster a widespread range of applications (150) including low-power spintronics, quantum computing, and optical communications.

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RESEARCH ARTICLE SUMMARY

NEURODEGENERATION

Heterochromatin anomalies and double-stranded RNA accumulation underlie *C9orf72* poly(PR) toxicity

Yong-Jie Zhang, Lin Guo, Patrick K. Gonzales, Tania F. Gendron, Yanwei Wu, Karen Jansen-West, Aliesha D. O'Raw, Sarah R. Pickles, Mercedes Prudencio, Yari Carlomagno, Mariam A. Gachechiladze, Connor Ludwig, Ruilin Tian, Jeannie Chew, Michael DeTure, Wen-Lang Lin, Jimei Tong, Lillian M. Daugherty, Mei Yue, Yuping Song, Jonathan W. Andersen, Monica Castanedes-Casey, Aishe Kurti, Abhishek Datta, Giovanna Antognetti, Alexander McCampbell, Rosa Rademakers, Björn Oskarsson, Dennis W. Dickson, Martin Kampmann, Michael E. Ward, John D. Fryer, Christopher D. Link, James Shorter, Leonard Petrucelli*

INTRODUCTION: Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are fatal neurodegenerative diseases that share clinical and neuropathological features. Furthermore, the most common genetic cause of both FTD and ALS is a GGGGCC (G_4C_2) repeat expansion in the *C9orf72* gene. This repeat expansion leads to several abnormalities, including *C9orf72* haploinsufficiency, the accumulation of repeat RNA, and the production of five aggregation-prone proteins composed of repeating dipeptides. However, the contribution of these abnormalities to disease pathogenesis remains unresolved.

RATIONALE: Among the five dipeptide repeat proteins nonconventionally translated from expanded G_4C_2 repeats, proline-arginine (PR) repeat proteins [poly(PR) proteins] have proven especially toxic in various model systems. Their involvement in *C9orf72*-associated

FTD and ALS (c9FTD/ALS) has nevertheless been questioned because poly(PR) pathology is relatively infrequent in c9FTD/ALS patient brains. Postmortem tissues, however, represent end-stage disease and do not necessarily reflect early events in the disease process. Therefore, we generated mice that express poly(PR) in the brain to evaluate the temporal consequences of its expression in a mammalian in vivo model. More specifically, we engineered mice to express green fluorescent protein (GFP)-conjugated (PR)₅₀ (a 50-repeat PR protein) or GFP via intracerebroventricular administration of adeno-associated viral vectors and then performed behavioral, pathological, and transcriptomic characterizations of poly(PR) mice in comparison with control GFP mice.

RESULTS: We found that ~60% of poly(PR)-expressing mice died by 4 weeks of age and had significantly decreased brain and body

weights at death compared with age-matched GFP control mice. Poly(PR) mice that escaped premature death developed motor and memory impairments, likely as a consequence of their progressive brain atrophy, neuron loss, loss of poly(PR)-positive cells, and gliosis. In investigating the mechanisms by which poly(PR) caused neurodegeneration and functional deficits, we found that poly(PR) localized to heterochromatin (highly condensed regions of transcriptionally silent chromatin) and caused abnormal histone H3 methylation, features

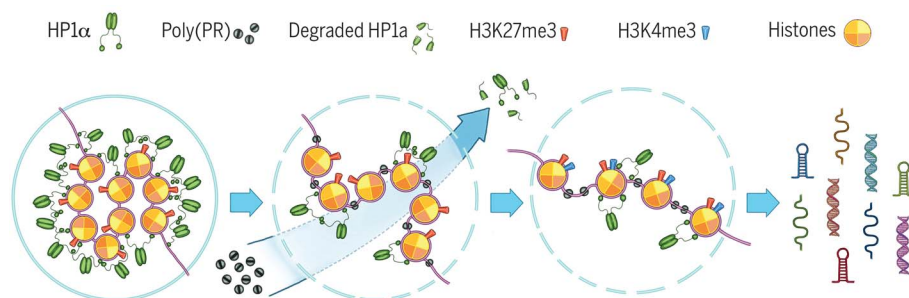
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that we also detected in brain tissues from patients with c9FTD/ALS. Additionally, we observed aberrations in nuclear lamins and heterochromatin protein 1 α (HP1 α), key proteins that maintain heterochromatin structure and regulate gene silencing. Nuclear lamina invaginations and decreased HP1 α protein expression were seen in poly(PR)-positive cells in poly(PR) mice, and in vitro studies demonstrated that poly(PR) disrupted HP1 α liquid phases. Because poly(PR)-induced histone H3 posttranslational modifications, lamin invaginations, and decreased HP1 α levels could profoundly affect gene expression, we compared transcriptome profiles between control and poly(PR) mice. As well as analyzing differentially expressed genes, we examined repetitive element expression given that repetitive DNA sequences make up a large portion of heterochromatin and that repetitive elements are substantially up-regulated in the brains of c9FTD/ALS patients. Whereas the majority of differentially expressed genes in poly(PR) mice were down-regulated, repetitive elements were markedly up-regulated, and this up-regulation was accompanied by the accumulation of double-stranded RNA. Furthermore, we confirmed that HP1 α depletion caused double-stranded RNA accumulation in human induced pluripotent stem cell-derived neurons and decreased their survival.

CONCLUSION: Our studies provide compelling evidence that, by disrupting HP1 α liquid phases, interacting with heterochromatin, and eliciting aberrant histone posttranslational modifications, poly(PR) adversely influences heterochromatin structure. Consequently, repetitive element expression is induced and double-stranded RNA accumulates, contributing to the neurodegeneration seen in patients with c9FTD/ALS. Rescuing histone methylation, lamin, and HP1 α abnormalities and/or inhibiting abnormal repetitive element expression may represent promising therapeutic strategies for treating c9FTD/ALS. ■

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Poly(PR) interactions with heterochromatin cause repetitive element expression.

Heterochromatin consists of tightly packed nucleosomes, DNA segments wound around histones. The *C9orf72*-associated dipeptide repeat protein poly(PR) disrupts HP1 α liquid compartments on heterochromatin, thus evicting HP1 α from heterochromatin and causing its degradation. Poly(PR) also binds heterochromatin and causes abnormal histone H3 methylation. These events alter heterochromatin structure and ultimately increase repetitive element expression and double-stranded RNA accumulation.

RESEARCH ARTICLE

NEURODEGENERATION

Heterochromatin anomalies and double-stranded RNA accumulation underlie *C9orf72* poly(PR) toxicity

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How hexanucleotide GGGGCC (G₄C₂) repeat expansions in *C9orf72* cause frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) is not understood. We developed a mouse model engineered to express poly(PR), a proline-arginine (PR) dipeptide repeat protein synthesized from expanded G₄C₂ repeats. The expression of green fluorescent protein-conjugated (PR)₅₀ (a 50-repeat PR protein) throughout the mouse brain yielded progressive brain atrophy, neuron loss, loss of poly(PR)-positive cells, and gliosis, culminating in motor and memory impairments. We found that poly(PR) bound DNA, localized to heterochromatin, and caused heterochromatin protein 1α (HP1α) liquid-phase disruptions, decreases in HP1α expression, abnormal histone methylation, and nuclear lamina invaginations. These aberrations of histone methylation, lamins, and HP1α, which regulate heterochromatin structure and gene expression, were accompanied by repetitive element expression and double-stranded RNA accumulation. Thus, we uncovered mechanisms by which poly(PR) may contribute to the pathogenesis of *C9orf72*-associated FTD and ALS.

Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), two fatal neurodegenerative diseases, share neuropathological features, such as TAR DNA-binding protein 43 (TDP-43) pathology, and clinical symptoms. FTD patients typically present with progressive changes in behavior, executive function, and/or language caused by frontal and temporal lobe degeneration but can also develop ALS-like motor symptoms. Patients

with ALS, which is caused by upper and lower motor neuron loss, develop muscle weakness, atrophy, and paralysis. In addition, ~50% of ALS patients experience cognitive and/or behavioral changes. The clinical and neuropathological overlap between FTD and ALS is accompanied by genetic overlap: A hexanucleotide GGGGCC (G₄C₂) repeat expansion in intron 1 of chromosome 9 open reading frame 72 (*C9orf72*) is the most common known genetic cause of FTD and ALS (1, 2). The mechanisms by which *C9orf72* G₄C₂ repeat expansions cause *C9orf72*-associated FTD and ALS (c9FTD/ALS) are being extensively investigated. Mounting evidence implicates both loss-of-function and gain-of-function mechanisms in c9FTD/ALS pathogenesis. For instance, loss of C9ORF72 causes immune dysregulation (3, 4) and impairs the autophagy-lysosome pathway (5–9), which may enhance abnormal protein deposition. The accumulation of expanded repeat-containing transcripts, conversely, is thought to cause toxic gains of function. These transcripts bind several RNA binding proteins and form RNA foci, thus impairing RNA metabolism (10–14), nucleocytoplasmic transport (15, 16), and RNA transport granule function (17). Moreover, these transcripts produce glycine-alanine (GA), glycine-

proline (GP), glycine-arginine (GR), proline-arginine (PR), and proline-alanine (PA) dipeptide repeat (DPR) proteins [poly(GA), poly(GP), poly(GR), poly(PR), and poly(PA)] through repeat-associated non-ATG translation (18–22). All five DPR proteins form neuronal inclusions in patients with c9FTD/ALS (18–22), but studies in cultured cells and neurons, as well as *Drosophila*, suggest that arginine-rich poly(PR) is the most toxic DPR protein (23–32). Several mechanisms have been ascribed to poly(PR)-induced toxicity, including nucleolar stress (23, 24, 26, 30) and impaired nucleocytoplasmic transport (27, 28), protein translation (26, 31), and stress granule dynamics (26, 30, 32). Although poly(PR) is considered highly toxic, poly(PR) pathology is infrequent in c9FTD/ALS patient brains (19, 20, 33, 34), raising questions about its contribution to c9FTD/ALS pathogenesis. However, because postmortem tissues represent end-stage disease and do not necessarily reflect early events in the disease process, we generated mice that express poly(PR) in the brain to evaluate the temporal consequences of poly(PR) expression in a mammalian in vivo model.

GFP-(PR)₅₀ mice developed neurodegeneration and behavioral deficits

We engineered mice to express green fluorescent protein (GFP)-conjugated (PR)₅₀ (a 50-repeat PR protein) or GFP in the brain via intracerebroventricular administration of adeno-associated virus serotype 1 (AAV1) at postnatal day 0. A codon-optimized vector was used to specifically express GFP-(PR)₅₀ in the absence of repeat RNA. Consistent with the reported toxicity of poly(PR) (23–32), ~60% of GFP-(PR)₅₀-expressing mice died by 4 weeks of age (fig. S1A) and had significantly decreased brain and body weights at death compared with age-matched GFP-expressing control mice (fig. S1, B to D). GFP-(PR)₅₀ mice that escaped premature death were sacrificed at 1 or 3 months of age for more in-depth analyses. These mice developed a progressive decrease in brain weight (fig. S2A), and hematoxylin- and eosin-stained brain sections revealed cortical thinning and reduced hippocampal volume in 3-month-old GFP-(PR)₅₀ mice compared with age-matched GFP mice (fig. S2B). With the exception of 3-month-old female mice, no difference in body weight was observed between age- and sex-matched GFP and GFP-(PR)₅₀ mice (fig. S2C).

Given that our gross morphological analysis revealed brain atrophy in GFP-(PR)₅₀ mice (fig. S2B), we examined the relationship between poly(PR) expression and neuron loss. Immunohistochemical staining showed a predominantly nuclear distribution of poly(PR) in the cortices and cerebellums of 1- and 3-month-old GFP-(PR)₅₀ mice (Fig. 1A and fig. S3A). Virtually all poly(PR)-positive cells were immunoreactive for the neuronal markers microtubule-associated protein 2 (MAP2) and NeuN, indicating that the poly(PR) expression was neuronal (fig. S3B). Notably, the number of poly(PR)-positive cells in the cortex and cerebellum significantly decreased from 1 to 3 months

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of age (Fig. 1, A and B, and fig. S3C). Consistent with these findings, immunoassay and immunoblot analyses showed a significant reduction in poly(PR) levels in 3-month-old GFP-(PR)₅₀ mice compared with 1-month-old GFP-(PR)₅₀ mice

(Fig. 1C and fig. S3, D and E). The age-dependent loss of poly(PR)-expressing cells in the cortex and cerebellum was accompanied by an age-dependent loss of NeuN-positive cortical neurons (Fig. 1, D and E) and of cerebellar Purkinje

cells (fig. S3, F and G), suggesting that poly(PR) expression caused cell-autonomous neuron death. Transgene RNA levels were significantly higher in GFP mice than in GFP-(PR)₅₀ mice, arguing against neuronal loss being caused by high transgene expression (fig. S3H).

Inflammation is believed to be a key process in FTD and ALS and is often associated with neurodegeneration. Hence, we examined the brains of mice for reactive astrocytes and microglia. Transcript levels of *Gfap*, a marker of astrogliosis, were significantly increased in the brains of GFP-(PR)₅₀-expressing mice compared with those of GFP-expressing mice at 3 months of age, whereas no significant difference was observed at 1 month of age (fig. S4A). Transcript levels of *CD68*, a marker of activated microglia, were elevated at 1 and particularly 3 months of age in GFP-(PR)₅₀ mice (fig. S4A). Similar to RNA levels of *Gfap* and *CD68*, Gfap and CD68 protein expression in the cortices and cerebellums of 3-month-old GFP-(PR)₅₀ mice increased significantly, as confirmed by immunohistochemical analysis (fig. S4, B to E).

Examination of c9FTD/ALS behavioral features in 3-month-old GFP- or GFP-(PR)₅₀-expressing mice revealed both motor and cognitive deficits in the latter group (Fig. 1, F and G). On the rotarod test, GFP-(PR)₅₀ mice exhibited a decreased latency to fall compared with GFP mice (Fig. 1F), indicating impaired motor skills. GFP-(PR)₅₀ mice also displayed an associative memory deficit, as evidenced by a decrease in cued, but not contextual, freezing in a fear-conditioning task (Fig. 1G).

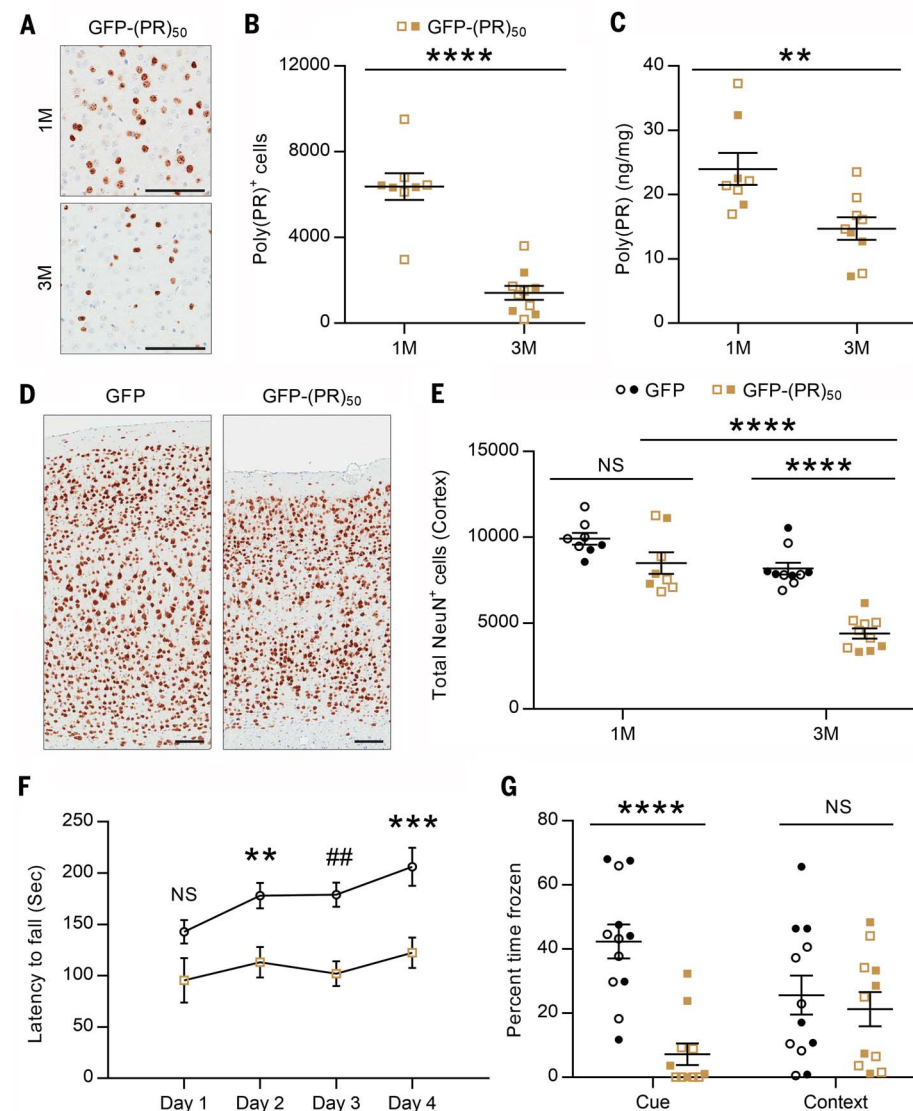


Fig. 1. GFP-(PR)₅₀ mice exhibited neurodegeneration and behavioral deficits. Immunohistochemical (A) and quantitative (B) analyses of anti-PR immunoreactivity in the cortices of GFP-(PR)₅₀ mice at 1 month (1M) ($n = 8$ mice) and 3 months (3M) ($n = 10$ mice) of age. Scale bars, 100 μ m. (C) An immunoassay was used to compare poly(PR) levels in cortex and hippocampus lysates of GFP-(PR)₅₀ mice at 1 ($n = 8$ mice) and 3 ($n = 9$ mice) months of age. (D) Representative images of NeuN-labeled cells in the cortices of 3-month-old GFP or GFP-(PR)₅₀ mice. Scale bars, 100 μ m. (E) Quantification of NeuN-labeled cells in the cortices of GFP mice at 1 ($n = 8$ mice) and 3 ($n = 10$ mice) months of age or GFP-(PR)₅₀ mice at 1 ($n = 8$ mice) and 3 ($n = 10$ mice) months of age. (F) Results from a 4-day rotarod test used to assess motor deficits of 3-month-old mice expressing GFP ($n = 12$ mice) or GFP-(PR)₅₀ ($n = 11$ mice) by evaluating their latency to fall from a rotating rod. (G) Results from the fear-conditioning test used to assess the associative learning and memory of 3-month-old mice expressing GFP ($n = 12$ mice) or GFP-(PR)₅₀ ($n = 11$ mice) by evaluating the percentage of time frozen in response to a conditioned (cued) or unconditioned (context) stimulus. Data are presented as the mean $\pm$ SEM. Male mice are represented by solid symbols, whereas female mice are represented by empty symbols. In (B) and (C), **** $P < 0.0001$, ** $P = 0.0072$, two-tailed unpaired t test. In (E), **** $P < 0.0001$; NS (not significant), $P = 0.1130$; two-way ANOVA and Tukey's post hoc analysis. In (F), NS, $P = 0.1269$; ** $P = 0.0096$; ## $P = 0.0015$; *** $P = 0.0005$; two-way ANOVA and Tukey's post hoc analysis. In (G), **** $P < 0.0001$; NS, $P = 0.6007$; two-tailed unpaired t test.

Poly(PR) proteins localized to heterochromatin and elicited aberrant posttranslational modifications of histone H3

Previous studies reported that poly(PR) causes cell death by accumulating in the nucleoli of cultured cells and neurons (23, 24, 26, 30). Our GFP-(PR)₅₀-expressing mice provided an opportunity to investigate the mechanisms that underlie poly(PR)-induced neurotoxicity in vivo. We began by examining the cellular localization of poly(PR) in the brains of mice and found it to be present in a punctate pattern throughout the nucleus (Fig. 2A). Whereas some GFP-(PR)₅₀ colocalized with the nucleolar markers nucleophosmin (NPM1) and fibrillarin (Fig. 2A), the majority of GFP-(PR)₅₀ formed punctate structures that colocalized with the DNA-staining dye Hoechst 33258, suggestive of a heterochromatic distribution of poly(PR). Immunoelectron microscopy revealed that anti-poly(PR) antibodies decorated heterochromatin in the cortices of 3-month-old GFP-(PR)₅₀ mice (Fig. 2B) and purified mouse genomic DNA incubated with synthetic (PR)₈ peptides (Fig. 2C). Furthermore, electron microscopy showed that incubating genomic DNA with (PR)₈ or (GR)₈ peptides, but not (GA)₈, (GP)₈, or (PA)₈ peptides, changed DNA morphology, suggesting an electrostatic interaction between the negatively charged DNA and the positively charged (PR)₈ and (GR)₈ (Fig. 2C and fig. S5A). Electrophoretic mobility

shift assays also demonstrated that single- and double-stranded DNA bound to (PR)₂₀ and (PR)₈ peptides, thus forming higher-molecular-weight, less mobile complexes that were not observed when DNA was incubated with (PA)₈ peptides (Fig. 2D and fig. S5B).

To confirm that poly(PR) localized to heterochromatin in mice, cortical sections from 3-month-

old GFP-(PR)₅₀ mice were co-stained for poly(PR) and the heterochromatin markers histone H3 lysine 9 trimethylation (H3K9me3) and histone H3 lysine 27 trimethylation (H3K27me3) or the euchromatin marker histone H3 lysine 4 trimethylation (H3K4me3) (Fig. 2E and fig. S5, C and D). These studies established that GFP-(PR)₅₀ essentially completely colocalized with hetero-

chromatin markers (Fig. 2E and fig. S5C). Whereas the euchromatin marker H3K4me3 expectedly failed to colocalize with Hoechst 33258-stained heterochromatin in poly(PR)-negative cells, it did colocalize with heterochromatin and poly(PR) in GFP-(PR)₅₀-positive cells (Fig. 2E and fig. S5D). Moreover, levels of both the transcriptionally repressive H3K27me3 and the transcriptionally active H3K4me3 were increased in cells expressing poly(PR) compared with poly(PR)-negative cells in mice expressing GFP-(PR)₅₀ or GFP, and this was especially apparent for Hoechst 33258-stained pericentromeric heterochromatin (fig. S5, C to F). These data suggest that poly(PR) influences histone H3 posttranslational modifications. As in GFP-(PR)₅₀ mice, poly(PR) colocalized with H3K27me3 and H3K4me3 in the cortices of c9FTD/ALS patients (Fig. 2F, fig. S5G, and table S1). In fact, all nuclear poly(PR) inclusions detected in the cortices of patients with c9FTD/ALS colocalized with H3K27me3 and H3K4me3.

Poly(PR) proteins caused nuclear lamina invaginations, reduced HP1 α protein expression, and disrupted HP1 α liquid phases

Next, we investigated the influence of poly(PR) on heterochromatin structure by examining the lamins and heterochromatin protein 1 α (HP1 α), key proteins in establishing and maintaining heterochromatin structure (35–38). Staining of mouse brain sections for lamins A/C and B showed a significantly higher frequency of nuclear lamina invaginations in cells expressing poly(PR) than in poly(PR)-negative cells in mice expressing GFP-(PR)₅₀ or GFP (Fig. 3A and fig. S6A). Moreover, we noted that cells expressing poly(PR) showed markedly decreased expression of HP1 α protein (Fig. 3B and fig. S6B) but not HP1 α mRNA (fig. S6C). Rather, a modest increase in HP1 α mRNA was observed, which may reflect a compensatory mechanism. By contrast to HP1 α , HP1 β remained unchanged in poly(PR)-positive cells (fig. S6D). Despite the finding that (GR)₈, like (PR)₈, changed DNA morphology in vitro (fig. S5A), HP1 α protein levels were not altered in GFP-(GR)₁₀₀ mice (fig. S6E), nor does poly(GR) influence lamin distribution in mice (39). Because poly(GR) does not localize to the nucleus, these data suggest that poly(PR)-induced alterations of lamins and HP1 α are caused by the heterochromatic distribution of poly(PR) within the nucleus.

Poly(PR) may cause decreases in HP1 α by impairing lamin function (40–42) and may also influence HP1 α more directly, given that both poly(PR) and HP1 α localize to heterochromatin. HP1 α undergoes liquid-liquid phase separation (LLPS), with liquid HP1 α compartments sequestering compacted chromatin and promoting heterochromatin-mediated gene silencing (43, 44). Poly(PR) interferes with the LLPS of RNA binding proteins with prionlike domains and promotes aberrant phase transitions from liquid droplets to solid aggregates (26). Thus, we examined whether poly(PR) disrupted preformed HP1 α liquid droplets in vitro to mimic in vivo conditions

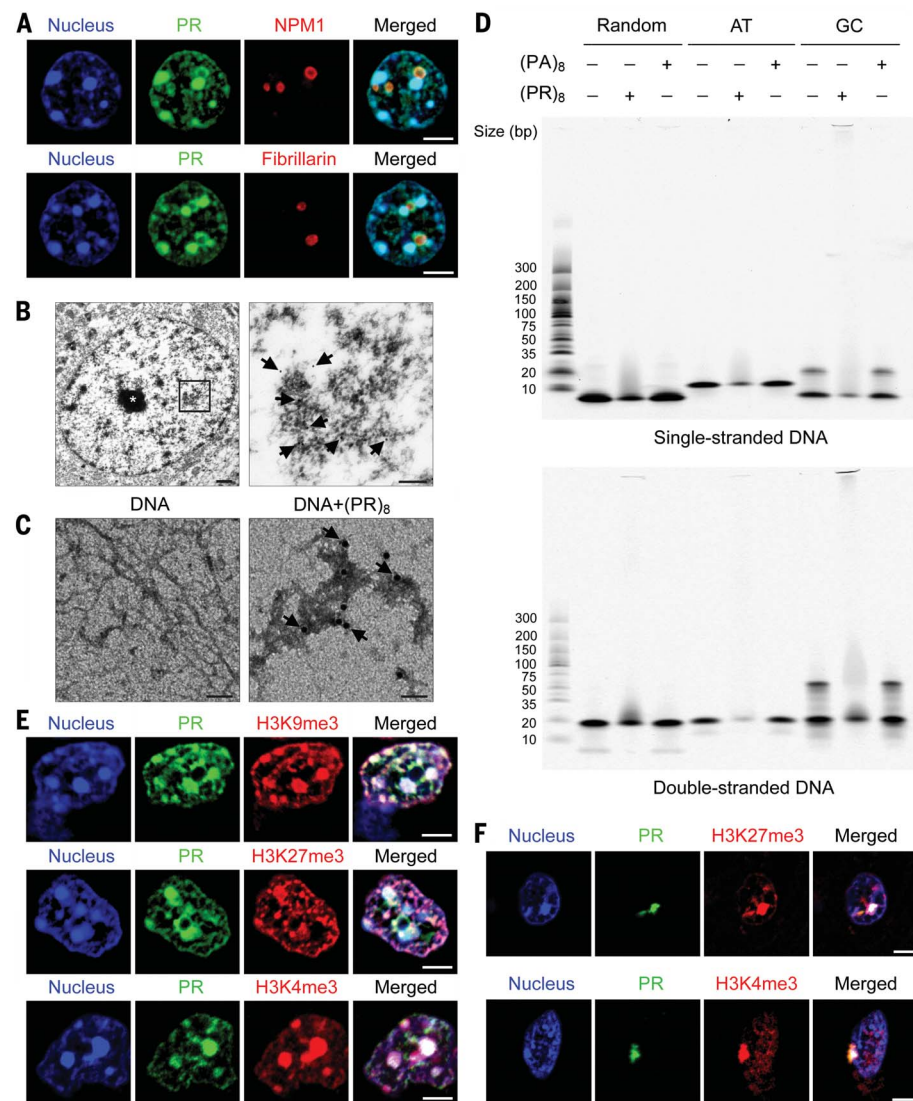


Fig. 2. Poly(PR) proteins localized to heterochromatin in GFP-(PR)₅₀ mice and c9FTD/ALS patients. (A) Double immunofluorescence staining for GFP-(PR)₅₀ and nucleolar markers (NPM1 and fibrillarin) in the cortices of 3-month-old GFP-(PR)₅₀ mice ($n = 5$). Scale bars, 5 μ m. (B) Immunoelectron microscopy using an anti-PR antibody labeled with gold particles in the cortices of 3-month-old GFP-(PR)₅₀ mice. The selected region in the low-magnification image (left) is shown at high magnification (right). * indicates the nucleolus. Arrows indicate gold particles. Scale bars, 0.5 μ m (left) and 0.2 μ m (right). (C) Immunoelectron microscopy analysis of purified mouse genomic DNA incubated with (PR)₈ peptide by using an anti-PR antibody labeled with gold particles. Arrows indicate gold particles. Scale bars, 50 nm. (D) Electrophoretic mobility shift assays using single- and double-stranded DNA co-incubated (+) or not (–) with (PR)₈ or (PA)₈ peptides. AT, AT-rich oligonucleotides; GC, GC-rich oligonucleotides. (E) Double immunofluorescence staining for GFP-(PR)₅₀ and heterochromatin (H3K9me3 and H3K27me3) or euchromatin (H3K4me3) markers in the cortices of 3-month-old GFP-(PR)₅₀ mice ($n = 7$). Scale bars, 5 μ m. (F) Double immunofluorescence staining for poly(PR) and H3K27me3 or H3K4me3 in the cortices of c9FTD/ALS patients. All nuclear poly(PR) inclusions colocalized with H3K27me3 ($n = 7$ cases) and with H3K4me3 ($n = 4$ cases). Representative images from case 4 are shown. See also fig. S5F and table S1. Scale bars, 5 μ m.

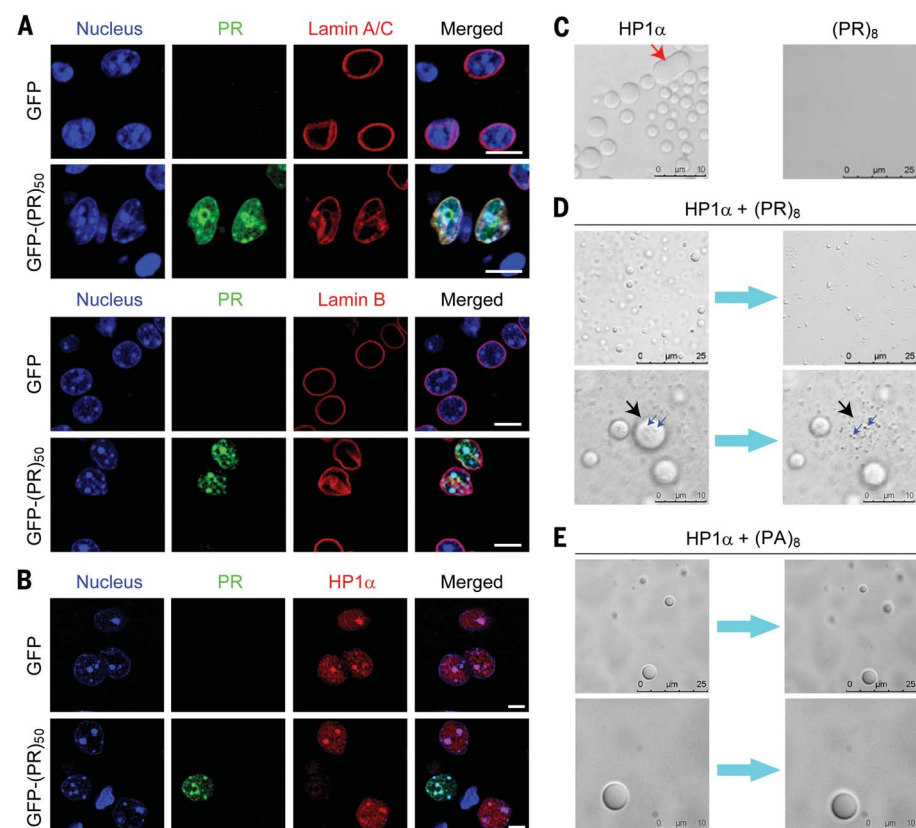


Fig. 3. Poly(PR) proteins caused lamin invaginations, reduced HP1 α levels, and disrupted HP1 α liquid droplets. (A) Double immunofluorescence staining for GFP-(PR)₅₀ and lamin A/C or lamin B in the cortices of 3-month-old GFP ($n = 11$) or GFP-(PR)₅₀ ($n = 7$) mice. Scale bars, 10 μ m. (B) Double immunofluorescence staining for GFP-(PR)₅₀ and HP1 α in the cortices of 3-month-old GFP-(PR)₅₀ mice ($n = 7$). Scale bars, 5 μ m. (C) Differential interference contrast (DIC) microscopy images of HP1 α droplets at a concentration of 90 μ M HP1 α before the addition of (PR)₈. The arrow indicates the fusing of two liquid droplets. By contrast, (PR)₈ peptides at a concentration of 245 μ M did not form droplets under these conditions. (D) DIC microscopy images of HP1 α droplets at 90 μ M HP1 α (top, low magnification; bottom, high magnification) after the addition of 245 μ M (PR)₈. Black arrows indicate the bursting of a preformed HP1 α droplet after the addition of (PR)₈. Small blue arrows indicate the solid components within HP1 α droplets. (E) DIC microscopy images of HP1 α droplets at 90 μ M HP1 α (top, low magnification; bottom, high magnification) after the addition of 245 μ M (PA)₈ peptides. In (D) and (E), turquoise arrows between images indicate progression over time.

when poly(PR) encounters liquid HP1 α compartments on heterochromatin. We first assembled HP1 α droplets, spherical structures that readily underwent fusion consistent with liquidlike properties (Fig. 3C, arrow). To these HP1 α liquid droplets, we added (PR)₈, which under these conditions did not phase separate in isolation (Fig. 3C). Initially, (PR)₈ rapidly induced the formation of solid components (Fig. 3D, blue arrows), which were not observed before the addition of (PR)₈ (Fig. 3C), inside the HP1 α liquid droplets. HP1 α droplets then violently burst to release these solid components (Fig. 3D, black arrows, and movie S1). After incubation with (PR)₈, few HP1 α droplets remained (Fig. 3D). By contrast, (PA)₈ had no effect on preformed HP1 α liquid droplets (Fig. 3E and movie S2). Thus, (PR)₈ but not (PA)₈ rapidly elicited an aberrant phase transition within HP1 α droplets and caused their rupture. We suggest that this mechanism directly

disrupts HP1 α liquid compartments on heterochromatin, thereby evicting and depleting HP1 α from heterochromatin, where it is replaced by poly(PR) in vivo.

Poly(PR) enhanced repetitive element expression and caused the accumulation of double-stranded RNA

Given that poly(PR) elicited aberrant post-translational modifications of histone H3 and caused lamin invagination and decreases in HP1 α protein, which are expected to influence gene expression, we compared transcriptome profiles between 3-month-old mice expressing GFP or GFP-(PR)₅₀. Clustering of the 1000 most variable genes showed distinct expression profiles for GFP-(PR)₅₀ and GFP mice (Fig. 4A). Of the 2196 genes that were differentially expressed in GFP-(PR)₅₀ mice, the majority (1552) were down-regulated (Fig. 4B and dataset S1). Weighted

gene coexpression network analysis of differentially expressed genes with a merging distance of 0.01 identified 13 modules, all of which were significantly associated with genotype after adjustment for multiple tests (Fig. 4C and dataset S1). Gene Ontology enrichment analysis across these modules identified the pink and blue modules as the most significantly enriched. Within the pink module, molecular function and biological process analyses, respectively, identified top terms specific to unfolded protein binding ($P = 2.13 \times 10^{-8}$; Bonferroni correction = 8.77×10^{-5}) and protein folding ($P = 3.05 \times 10^{-9}$; Bonferroni correction = 3.63×10^{-5}). Within the blue module, molecular function, cellular component, and biological process analyses, respectively, identified top terms specific to the structural constituent of the ribosome ($P = 9.26 \times 10^{-8}$; Bonferroni correction = 3.81×10^{-4}), ribosome biogenesis ($P = 1.86 \times 10^{-6}$; Bonferroni correction = 2.21×10^{-2}), and the ribosomal subunit ($P = 2.26 \times 10^{-6}$; Bonferroni correction = 3.75×10^{-3}). These pathways have been implicated in c9FTD/ALS (26, 31, 39, 45, 46).

In addition to identifying differentially expressed genes in GFP-(PR)₅₀ mice, we examined the expression of repetitive elements (REs) given our findings that poly(PR) localized to heterochromatin and that repetitive DNA sequences, which make up a large portion of heterochromatin (47), are significantly up-regulated in the brains of patients with c9ALS (48). By using a combination of RepEnrich2 and DESeq2, we identified a total of 1067 RepeatMasker-derived REs belonging to multiple repeat classes (dataset S2). Notably, the proportions of REs in distinct classes were comparable among GFP, GFP-(PR)₅₀, and GFP-(GR)₁₀₀ mice (table S2). In GFP-(PR)₅₀ mice compared with control GFP mice, 172 REs were differentially expressed [false discovery rate (FDR) < 0.10], with ~92% of these being up-regulated (Fig. 5A). This marked up-regulation of REs contrasted with the down-regulation of the majority of differentially expressed genes (Fig. 4B). Quantitative polymerase chain reaction (qPCR) analysis of select RE hits confirmed their up-regulation (Fig. 5B and fig. S7A). No specific classes of REs were enriched, indicating a global change in their expression. Of note, no RE was differentially expressed in mice expressing GFP-(GR)₁₀₀ (fig. S7B). Because RE transcripts were elevated in GFP-(PR)₅₀ mice and because these transcripts can form double-stranded RNA (dsRNA) (49–51), we evaluated whether dsRNA was produced in GFP-(PR)₅₀ mice. Immunofluorescence staining with an antibody against dsRNA revealed that dsRNA was specifically increased in cells expressing poly(PR) (Fig. 5C and fig. S7C). Accumulation of dsRNA was not observed in mice expressing GFP-(GR)₁₀₀ (fig. S7D).

A previous study found that the deletion of HPL-2, a *Caenorhabditis elegans* ortholog of HP1 α , leads to the accumulation of dsRNA (52). To further determine whether the reduction of HP1 α in GFP-(PR)₅₀ mice contributed to the observed accumulation of dsRNA, we repressed HP1 α transcription in human cells by CRISPR interference

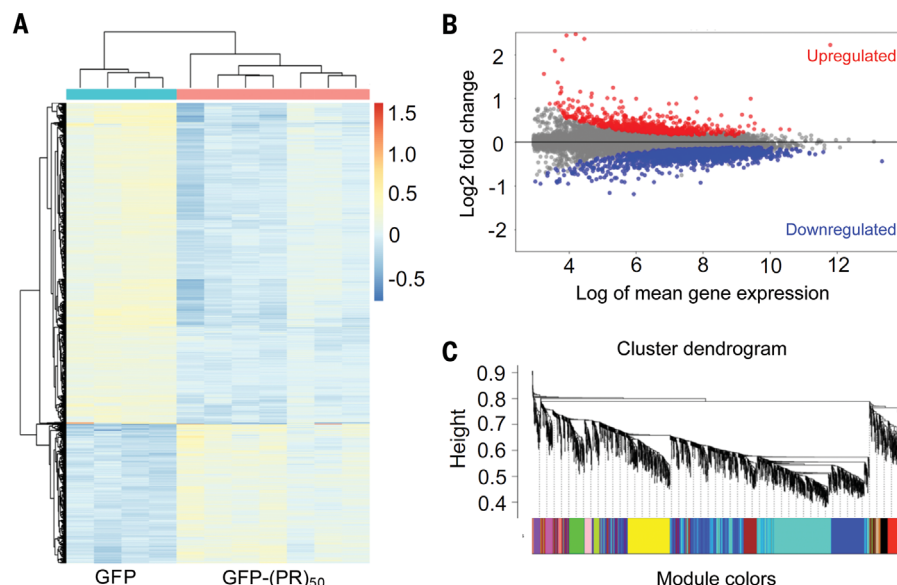


Fig. 4. Transcriptome alterations were identified in the brains of mice expressing GFP-(PR)₅₀. (A) Hierarchical clustering of the 1000 most variable genes between 3-month-old GFP mice ($n = 4$) and GFP-(PR)₅₀ mice ($n = 7$). (B) MA plots of up- and down-regulated genes (FDR < 0.01) in the cortices and hippocampi of 3-month-old mice expressing GFP-(PR)₅₀ ($n = 7$) compared with those of GFP controls ($n = 4$). The MA plot is based on the Bland-Altman plot, where M represents the log₂ fold change (y axis) and A represents the log of mean gene expression (x axis). (C) Gene modules identified in brains of 3-month-old mice expressing GFP-(PR)₅₀ ($n = 7$ mice) through weighted gene coexpression correlation network analyses using differentially expressed genes.

(CRISPRi), which is highly specific compared with RNA interference-based approaches (53, 54). To this end, we individually transduced human induced pluripotent stem cells (iPSCs) stably expressing nuclease-deactivated CRISPR-associated protein 9 fused to blue fluorescent protein and the KRAB repressor domain (dCas9-BFP-KRAB) (55) with five single guide RNAs (sgRNAs) predicted to mediate CRISPRi of the HP1 α gene. Because sgRNA 1 had the greatest ability to decrease HP1 α protein expression (fig. S7E), we differentiated dCas9-BFP-KRAB iPSC cells expressing HP1 α sgRNA 1 into neurons. HP1 α sgRNA 1 caused a ~40% reduction of HP1 α RNA, as assessed by qPCR (fig. S7F). Notably, dsRNA accumulated only in iPSC-differentiated neurons depleted of HP1 α (Fig. 5D). Moreover, cells positive for dsRNA showed immunoreactivity for active caspase-3 (Fig. 5E), suggesting that dsRNA is toxic to neurons.

Poly(PR) caused abnormalities in nucleocytoplasmic transport proteins

Earlier studies have demonstrated that poly(PR) aberrantly affects nuclear pores and impairs nucleocytoplasmic transport in cultured cells and yeast models (27, 28). Because poly(PR) caused lamin invagination in poly(PR) mice (Fig. 3A and fig. S6A), which may affect nuclear membrane integrity, we investigated whether poly(PR) also perturbs the nucleocytoplasmic transport factor Ran guanosine triphosphatase-activating protein 1 (RanGAP1) and nuclear pore complex (NPC) proteins, the latter assessed by using an antibody

that recognizes Phe-x-Phe-Gly (where x is usually a small residue such as Ser, Gly, or Ala) nucleoporin repeats. We observed abnormal nuclear membrane invaginations immunopositive for RanGAP1 or NPC proteins in poly(PR)-positive cells compared with poly(PR)-negative cells in mice expressing GFP-(PR)₅₀ or GFP (Fig. 6 and fig. S8A). RanGAP1 was abnormally distributed in virtually all poly(PR)-positive cells (Fig. 6A and fig. S8A), whereas NPC protein abnormalities in poly(PR)-positive cells were less frequent (Fig. 6B).

Given that defects in RanGAP1 may impair the nucleocytoplasmic transport of TDP-43 and promote its cytoplasmic accumulation, we evaluated whether poly(PR) caused TDP-43 pathology, a hallmark feature of c9FTD/ALS. However, similar to mice expressing poly(GA) or poly(GR) (39, 56), poly(PR)-expressing mice did not develop TDP-43 inclusions, suggesting that the expression of individual DPR proteins is insufficient to cause TDP-43 pathology within the time frames examined (Fig. 6C and fig. S8B). Additional pathological mechanisms associated with poly(PR) include stress granule formation and nucleolar stress (23, 24, 26, 30). Nevertheless, no evidence of stress granules was seen in the brains of GFP-(PR)₅₀ mice (fig. S8C). This may be due to the absence of cytoplasmic poly(PR) inclusions, which, similar to poly(GR) cytoplasmic inclusions, have been shown to initiate stress granule formation and sequester stress granule-associated proteins (26, 39). Likewise, although nucleolar poly(PR) was occasionally observed in GFP-(PR)₅₀ mice, no sign of nucleolar stress (i.e.,

repressed ribosomal RNA expression) was detected (fig. S8, D and E), suggesting that nucleolar poly(PR) levels must reach a threshold to induce nucleolar stress.

Discussion

In this study, we found that poly(PR) expression in the brain caused premature death in ~60% of mice, with surviving GFP-(PR)₅₀ mice developing age-dependent brain atrophy and neuron loss, as well as impaired motor and memory functions. GFP-(PR)₅₀ mice that succumbed to an early death exhibited higher poly(PR) levels than surviving mice (37.89 ± 11.88 versus 23.99 ± 7.07 ng/mg; $P = 0.0367$, two-tailed unpaired t test), indicating that poly(PR) toxicity is dose dependent. The age-dependent neuron loss in surviving mice was accompanied by a similar age-dependent loss of poly(PR)-positive cells, suggesting that poly(PR)-positive neurons progressively degenerated. These data are consistent with the results of a study showing that poly(PR) expression causes cultured neurons to die in a time-dependent fashion (24) and may also explain, at least in part, why poly(PR) pathology is rare in post-mortem brain tissues from c9FTD/ALS patients (19, 20, 33, 34), which reflect the end stage of disease.

The neurodegeneration and behavioral deficits of GFP-(PR)₅₀ mice were associated with the localization of poly(PR) to heterochromatin, highly condensed regions of transcriptionally silent chromatin (47). A heterochromatic localization of poly(PR) was also observed in c9FTD/ALS patients. Both increased H3K27me₃, which represses gene expression, and increased H3K4me₃, which activates gene expression, were observed in the heterochromatin of poly(PR)-expressing cells. Although the mechanism(s) by which poly(PR) elicited aberrant posttranslational modifications of histone H3 remain to be determined, these data suggest that poly(PR) causes epigenetic changes, which may influence heterochromatin function in c9FTD/ALS. Our RNA-sequencing (RNA-seq) and qPCR analyses of GFP-(PR)₅₀ mouse brain tissues revealed that RE sequences, which are enriched in heterochromatin DNA, were significantly up-regulated. Poly(PR)-induced RE expression in cultured cells was also evident through the accumulation of dsRNA, which can be formed by REs (49–51). The expression of REs, which is observed in several neurodegenerative diseases, is associated with neurotoxicity (49, 57, 58). Increased RE expression (48) and dsRNA accumulation (57) occur in c9FTD/ALS patients, and we reported previously that increases in general transcription may contribute to this enhanced RE expression (48). It is thus noteworthy that despite the marked up-regulation of REs in GFP-(PR)₅₀ mice, the majority of differentially expressed genes were down-regulated. Therefore, data from our GFP-(PR)₅₀ mice suggest that poly(PR) plays a role in RE expression in c9FTD/ALS but does so through heterochromatin alterations rather than enhanced transcription.

To more thoroughly evaluate how poly(PR) causes abnormal RE expression, we investigated

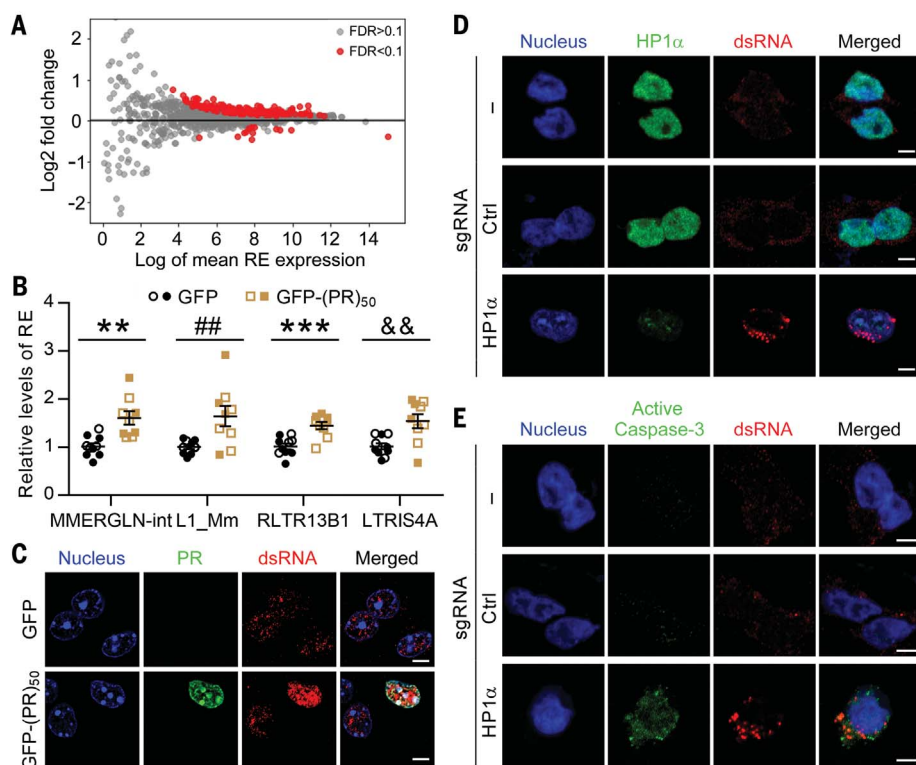


Fig. 5. Poly(PR) caused abnormal expression of REs and dsRNA accumulation. (A) MA plots of RNA-seq data show up- and down-regulated REs in the cortices and hippocampi of 3-month-old GFP-(PR)₅₀ mice (n = 7) compared with GFP mice (n = 4). Red dots represent the REs with a significant change (FDR < 0.10). (B) Validation of REs identified by RNA-seq in the cortices and hippocampi of 3-month-old GFP (n = 10) or GFP-(PR)₅₀ (n = 9) mice by qPCR. (C) Double immunofluorescence staining for GFP-(PR)₅₀ and dsRNA in the cortices of 3-month-old GFP mice (n = 3) or GFP-(PR)₅₀ mice (n = 7). Scale bars, 5 μm. (D) Double immunofluorescence staining for HP1α and dsRNA in human dCas9-iPSC-differentiated neurons stably expressing HP1α sgRNA 1. Scale bars, 5 μm. Ctrl, control. (E) Double immunofluorescence staining for active caspase-3 and dsRNA in human dCas9-iPSC-differentiated neurons stably expressing HP1α sgRNA 1. Scale bars, 5 μm. Data are presented as the mean ± SEM. Male mice are represented by solid symbols, whereas female mice are represented by empty symbols. In (B), **P = 0.0015; ##P = 0.0065; ***P = 0.0004; and &&P = 0.0036; two-tailed unpaired t test.

lamins and HP1α, key proteins in establishing and maintaining heterochromatin structure and in regulating gene silencing (35–38). Lamin dysfunction and/or loss of HP1α causes heterochromatin relaxation and mitigates gene silencing, which result in the increased expression of DNA REs within heterochromatin regions (35–38). It is thus of interest that the localization of poly(PR) to heterochromatin coincided with lamin invaginations and decreased HP1α expression in the brains of GFP-(PR)₅₀ mice. Whereas poly(PR) may cause HP1α depletion indirectly by inducing lamin dysfunction (40–42), it may also directly disrupt HP1α liquid compartments on heterochromatin, thereby evicting HP1α and rendering it vulnerable to degradation. Our data raise the following possible scenario: poly(PR) ruptures HP1α liquid phases on heterochromatin, interacts directly with DNA, and accumulates on heterochromatin. In turn, the disruption of HP1α liquid phases leads to lamin invaginations and HP1α depletion, which cause increased RE expression and dsRNA accumulation. In sup-

port of this, we confirmed that the knockdown of HP1α in human iPSC-differentiated neurons resulted not only in the accumulation of dsRNA but also in caspase-3 activation, a marker of apoptosis.

Although further studies are needed to delineate the precise mechanism by which poly(PR) causes lamin invaginations, one potential cause or consequence is defects in nucleocytoplasmic transport proteins, which have previously been implicated in c9FTD/ALS (15, 28, 56). We observed that RanGAP1 was abnormally distributed in all poly(PR)-positive cells in GFP-(PR)₅₀ mice, and NPC protein abnormalities were also found, albeit less frequently. The exact contribution of these phenomena to the neurodegeneration and behavioral deficits of GFP-(PR)₅₀ mice remains to be resolved.

Overall, our studies provide compelling evidence that, by interacting with DNA, eliciting aberrant histone posttranslational modifications, and disrupting HP1α liquid phases, poly(PR) adversely influences heterochromatin structural

organization. Consequently, RE expression is induced and dsRNA accumulates, contributing to the neurodegeneration seen in patients with c9FTD/ALS. Rescuing histone methylation, lamin, and HP1α abnormalities and/or inhibiting the abnormal expression of REs may represent promising therapeutic strategies for treating c9FTD/ALS.

Materials and methods summary

Detailed materials and methods can be found in the supplementary materials.

Generation of plasmids

To generate the AAV-GFP-(PR)₅₀ plasmid, a pEGFP-C1-(PR)₅₀ plasmid produced in our previous study (59) was subcloned into a modified AAV packaging vector containing the cytomegalovirus (CMV)-enhanced chicken β-actin promoter and the enhanced GFP (EGFP) coding sequence. To generate the HP1α-His plasmid, cDNA of an HP1α fragment was ligated to the NdeI and XhoI restriction sites of a pET-30a vector (69909-3, EMD Millipore). To generate Lenti-HP1α-sgRNA plasmids, we chose five sgRNA sequences against HP1α (CBX5) described in a previous study (60). The forward and reverse oligonucleotides were annealed and then ligated to the BstXI and BlnI restriction sites of a pU6-sgRNA EF1Alpha-puro-T2A-BFP vector (53). The sequence of GFP-(PR)₅₀ and primer sequences for cloning are listed in tables S3 and S4, respectively.

Virus production

To produce recombinant AAV1 (rAAV1) virus, AAV vectors expressing GFP or GFP-(PR)₅₀ were cotransfected with helper plasmids in human embryonic kidney (HEK) 293T cells. Cells were harvested 72 hours after transfection and lysed in the presence of 0.5% sodium deoxycholate (SDS) by freeze thawing, and the virus was isolated by using a discontinuous iodixanol gradient. The genomic titer of each virus was determined by qPCR. To produce lentivirus, HEK293T cells were cotransfected with plasmids of HP1α-sgRNA, psPAX2 (12260, Addgene), pMD2.G (12259, Addgene), and pAdVantage (E1711, Promega) by using Lipofectamine 2000 (11668-019, Thermo Fisher Scientific). Media containing virus were harvested, filtered, and used to transduce dCas9-iPSCs.

Approvals

All procedures using mice were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (61) and approved by the Mayo Clinic Institutional Animal Care and Use Committee (protocol number A42014).

Neonatal viral injections

Intracerebroventricular injections of virus in postnatal-day-0 C57BL/6J pups were performed as previously described by using 2 μl (1 × 10¹⁰ genomes/μl) of AAV1-GFP or AAV1-GFP-(PR)₅₀ solution per cerebral ventricle (39, 56).

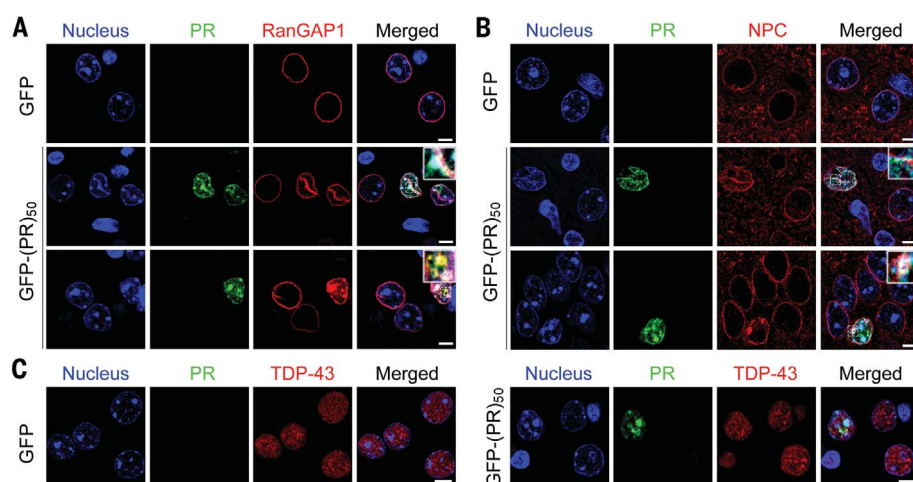


Fig. 6. Expression of GFP-(PR)₅₀ in mice caused abnormalities of RanGAP1 and NPC proteins but did not lead to TDP-43 pathology. (A) Double immunofluorescence staining for GFP-(PR)₅₀ and RanGAP1 in the cortices of 3-month-old GFP ($n = 11$) or GFP-(PR)₅₀ ($n = 7$) mice. Scale bars, 5 μ m. (B) Double immunofluorescence staining for GFP-(PR)₅₀ and NPC proteins in the cortices of 3-month-old GFP or GFP-(PR)₅₀ mice ($n = 4$ mice per group). Scale bars, 5 μ m. Insets in (A) and (B) show boxed areas at higher magnification. (C) Double immunofluorescence staining for GFP-(PR)₅₀ and TDP-43 in the cortices of 3-month-old GFP or GFP-(PR)₅₀ mice ($n = 3$ mice per group). Scale bars, 5 μ m.

Behavioral tests

Three-month-old mice expressing GFP ($n = 12$ mice) or GFP-(PR)₅₀ ($n = 11$ mice) were subjected to behavioral analysis in two consecutive weeks by the Mayo Mouse Behavior Core. On two consecutive days of week 1, mice were subjected to tests of contextual and cued fear conditioning. On four consecutive days of week 2, the mice were subjected to the rotarod test. A detailed description of these tests is provided in the supplementary materials.

Tissue processing

For RNA, protein, and immunostaining analyses, mice were euthanized by CO₂ and brains were harvested and cut sagittally across the midline. Sagittal half brains were immersion fixed in 10% formalin, embedded in paraffin, sectioned (to 5 μ m thick), and mounted on glass. The cortex and hippocampus of the other half brain were dissected and frozen on dry ice. Frozen mouse cortex and hippocampus tissues were homogenized in ice-cold tris-EDTA (TE), pH 8.0, with 2 $\times$ protease and phosphatase inhibitors. Homogenates were used for RNA or protein extractions. For immunoelectron microscopy studies, mice were anesthetized with sodium ketamine and xylazine and then perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were harvested, cut sagittally across the midline, and placed in 4% paraformaldehyde.

Preparation of brain protein lysates

To prepare protein lysates, 10% Triton X-100 and 10% SDS were added to brain homogenates at final concentrations of 1% and 2%, respectively. Homogenates were sonicated on ice and then centrifuged at 16,000 $\times$ g for 20 min. Super-

natants were collected as lysates, and protein concentrations were determined by a bicinchoninic acid (BCA) assay (23225, Thermo Fisher Scientific). Protein lysates were used for Western blot and immunoassay analyses as described in the supplementary materials by using antibodies listed in table S5.

Human tissues

Postmortem frontal cortical tissues from FTD and ALS patients with *C9orf72* repeat expansions were obtained from the Mayo Clinic Florida Brain Bank. Information on human patients is provided in table S1. Written informed consent was obtained from all subjects or their legal next of kin if they were unable to give written consent, and biological samples were obtained with Mayo Clinic Institutional Review Board approval.

Immunohistochemistry and immunofluorescence staining

Paraffin sections of mouse and human brains were subjected to immunohistochemistry and immunofluorescence staining as previously described (39, 56). Detailed methods for staining conditions, information on primary antibodies used, and quantification of neuropathology are provided in the supplementary materials and table S5.

Electrophoretic mobility shift assays

To analyze the binding of (PR)₂₀, (PR)₈, or (PA)₈ to single- or double-stranded DNA, Cy3-labeled random, CG-rich, or AT-rich oligonucleotides were incubated with the peptides before samples were resolved on 4 to 20% tris-borate EDTA (TBE) gels (Invitrogen) as described in the supplementary materials.

Postembedding immunoelectron microscopy

To examine the localization of poly(PR) proteins in mouse brain, immunoelectron microscopy was performed (56, 59). Thin sections were pretreated with sodium citrate buffer, and then rabbit polyclonal poly(PR) antibody (1:50) was used as the primary antibody and goat anti-rabbit immunoglobulin G (IgG) conjugated with 18-nm colloidal gold particles (1:20, Jackson ImmunoResearch Laboratories) was used as the secondary antibody. Thin sections stained with uranyl acetate and lead citrate were examined with a Philips 208S electron microscope (FEI) fitted with a Gatan 831 Orius charge-coupled device (CCD) camera.

RNA extraction, reverse transcription, and qPCR

To extract total RNA in mouse brain, 1 volume of brain homogenate was mixed with 3 volumes of Trizol LS reagent (10296010, Thermo Fisher Scientific) and frozen on dry ice. One day later, total RNA was extracted by using the Direct-zol RNA MiniPrep kit (R2073, Zymo Research). To extract total RNA from iPSC-differentiated neurons, cell pellets were lysed in TRI reagent and then total RNA was extracted by using the Direct-zol RNA MiniPrep kit. cDNA was obtained by reverse transcription PCR by using 1000 ng of RNA with random primers and a high-capacity cDNA transcription kit (4368814, Applied Biosystems). To quantify RNA levels of the indicated transcripts in mouse brain or cultured cells, qPCR was conducted as described in the supplementary materials. Primer sequences are listed in table S6. Relative RNA expression levels of *Gfap*, *CD68*, and *HPIa* were normalized to *Gapdh* or *GAPDH* values (endogenous transcript controls).

RNA-seq, Gene Ontology, and repetitive element analyses

The library preparation and RNA-seq were performed by the Mayo Clinic Sequencing Core Facility (Rochester, MN) as previously described (39) and as detailed in the supplementary materials. After the preparation of the libraries, samples were subjected to quality control, cluster generation, and sequencing on the Illumina HiSeq 2000 platform. All reads had a read length of 100 bp and were paired-end. The number of reads per sample is listed in table S7. Weighted gene coexpression network analysis, the Gene Ontology overrepresentation test, differential expression analysis, and RE analysis were performed as described in the supplementary materials.

Purification of recombinant HPIa proteins

HPIa-His plasmid was used for transformation in Rosetta(DE3)pLysS competent cells (709563, EMD4Biosciences). To induce the expression of recombinant proteins, bacteria were cultured overnight at 16°C in the presence of 0.5 mM isopropyl β -D-1-thiogalactopyranoside. After centrifugation, the bacterial pellet was washed, lysed, sonicated, and centrifuged. The resulting supernatant was applied to a HisTrap HP histidine-tagged protein purification column (17524801,

GE Healthcare), and the recombinant proteins were eluted, desalted, and concentrated.

In vitro DPR protein and HP1 α assays

HP1 α liquid droplets (90 μ M monomer) were formed in buffer containing 50 mM Tris, pH 7.5. The preformed HP1 α droplets were spotted onto a coverslip and imaged for 5 min, and then 245 μ M (PR) $_8$ or (PA) $_8$ peptides were added to the sample and the sample was imaged for another 5 to 10 min.

i^3 N iPSC culture and neuronal differentiation

We modified a well-characterized control iPSC line (WTC11) that harbors a dox-inducible NGN2 transgene at the AAVS1 locus (i^3 N) (62, 63). dCas9-BFP-KRAB was stably expressed in these i^3 N iPSCs via TALEN-mediated integration of a CAG-dCas9-BFP-KRAB expression cassette into the CLYBL safe harbor locus (55). The dCas9-BFP-KRAB iPSCs were transduced with lentivirus expressing HP1 α -sgRNA for 3 days and then selected by the addition of puromycin. To differentiate i^3 N dCas9-BFP-KRAB iPSCs expressing HP1 α sgRNA into neurons, iPSCs were dissociated by using Accutase (#AT-104, Innovative Cell Technologies) and then seeded onto dishes coated with Matrigel (354230, Corning). Three days after differentiation, cells were dissociated by using Accutase and then seeded onto poly-L-ornithine-coated plates (6-well plate) or glass coverslips (24-well plate) at a density of 7×10^5 or 2×10^4 cells per well, respectively. Six days later, the neurons were fixed with 4% paraformaldehyde for immunofluorescence staining or harvested for Western blot and qPCR analyses.

Statistics

Data are presented as the mean $\pm$ the standard error of the mean (SEM) and analyzed with a two-tailed unpaired *t* test or one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis (Prism statistical software). End points of interest [i.e., body weight, brain weight, poly(PR)-positive cells, poly(PR) expression, NeuN-positive cortical neurons, Purkinje cell density, transgene RNA levels, *Gfap* and *CD68* mRNA expression, and *Gfap* and *CD68* immunopositivity] were compared between male and female mice within each cohort. Other than body weight, no sex differences were observed. With the exception of body weight, analyses were performed on all mice within a given cohort. Data are presented such as to distinguish male and female mice; male mice are represented by solid symbols on dot plots, and female mice by empty symbols. Statistical analysis of RNA-seq data is described in the section RNA-seq, Gene Ontology, and RE analyses.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/eaav2606/suppl/DC1
Materials and Methods
Figs. S1 to S8
Tables S1 to S7
References (64–67)
Movies S1 and S2
Datasets S1 and S2

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RESEARCH ARTICLE SUMMARY

STRUCTURAL BIOLOGY

Recognition of the amyloid precursor protein by human γ -secretase

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INTRODUCTION: Alzheimer's disease (AD) is characterized by amyloid plaques in the brains of patients. The primary components of amyloid plaques are β -amyloid peptides ($A\beta$ s), which are derived from the amyloid precursor protein (APP). APP is first cleaved by α - or β -secretase to generate an 83- or 99-residue transmembrane (TM) fragment (APP-C83 or APP-C99), respectively. APP-C99 is then cleaved by the intramembrane aspartyl protease γ -secretase to generate the peptides $A\beta$ 48, $A\beta$ 45, $A\beta$ 42, and $A\beta$ 38 or $A\beta$ 49, $A\beta$ 46, $A\beta$ 43, and $A\beta$ 40. Of these, $A\beta$ 42 and $A\beta$ 43 are particularly prone to aggregation and formation of the amyloid plaques. Another substrate of γ -secretase is the Notch receptor. $A\beta$ oligomers may contribute to AD development. Therefore, inhibition of γ -secretase represents a potential therapeutic treatment for AD. Unfortunately, γ -secretase inhibitors caused severe side effects without any clear clinical benefits for AD

patients, perhaps owing to their inhibition of Notch cleavage.

Human γ -secretase comprises four subunits: presenilin (PS), PEN-2, APH-1, and nicastrin. As the catalytic subunit of γ -secretase, presenilin has two isoforms (PS1 and PS2). PS1—and, to a lesser extent, PS2 and APP—are frequently targeted for mutations in familial AD patients. Although free γ -secretase has been structurally characterized, how it recognizes APP remains largely unknown.

RATIONALE: Structural comparison of APP and Notch recognition by γ -secretase may reveal differences that can be exploited toward the design of substrate-specific inhibitors. However, the γ -secretase–substrate complex is extremely transient and has defied all efforts of isolation for structural studies. To this end, we developed a cross-linking strategy that involves mutation of two specific residues to

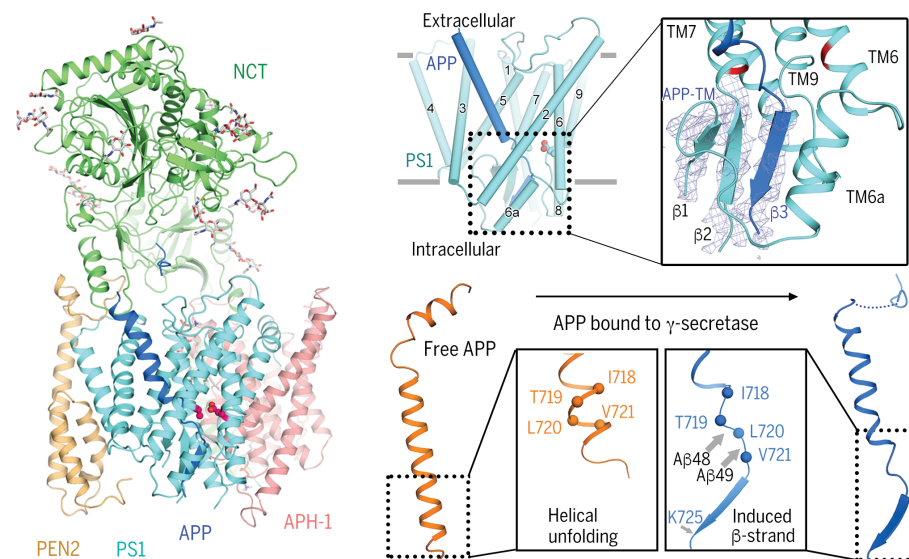
Cys and thus allows formation of a disulfide bond between PS1 and the substrate. Using this approach, we obtained a cross-linked complex between a variant of human γ -secretase [with PS1-Q112C (Gln¹¹²→Cys)] and APP-C83 (V695C). To avoid substrate cleavage, the catalytic residue Asp³⁸⁵ in PS1 was mutated to Ala. Because PS1 undergoes autoproteolysis during γ -secretase assembly to produce an N-terminal fragment (NTF) and a C-terminal

fragment (CTF), these two fragments of PS1 in the γ -secretase were coexpressed. The final γ -secretase contains PS1 (NTF-Q112C, CTF-D385A), PEN-2, APH-

1aL (a specific isoform of APH-1), and nicastrin. This γ -secretase was cross-linked to APP-C83 (V695C), and the complex was analyzed by cryo-electron microscopy (cryo-EM).

RESULTS: The cryo-EM structure of the cross-linked human γ -secretase–APP-C83 complex was determined at an average resolution of 2.6 Å. The quality of the EM map allows unambiguous identification of the bound APP fragment, which traverses through the center of the γ -secretase TM domain. Compared to substrate-free γ -secretase, the flexible transmembrane helix 2 (TM2) of PS1 becomes ordered upon binding to APP-C83 and contributes to its recognition, and the C-terminal portion of TM6 of PS1 is unraveled into a rigid loop followed by a short α helix (designated as TM6a). The TM of APP closely interacts with five surrounding TMs (TM2, TM3, TM5, TM6, and TM7) of PS1. Notably, the APP sequences on the C-terminal side of the TM form a β strand, which, together with two APP-induced β strands of PS1, constitutes a hybrid β sheet on the intracellular side. This β sheet guides γ -secretase to the scissile peptide bond of APP just preceding the N terminus of the β strand. Mutations that compromise the hybrid β sheet result in abrogation of APP cleavage by γ -secretase. Notably, residues at the interface between PS1 and APP are heavily targeted by recurring AD mutations.

CONCLUSION: The structure of human γ -secretase bound to APP-C83 constitutes a framework for understanding the function and disease relevance of γ -secretase. This structure, together with that of γ -secretase bound to Notch, reveals contrasting features of substrate recognition, which may be applied toward the design of substrate-specific inhibitors. ■



The cryo-EM structure of human γ -secretase bound to APP at 2.6-Å resolution. (Left)

Overall structure of the γ -secretase–APP-C83 complex. PS1, cyan; PEN-2, yellow; APH-1, pink; nicastrin (NCT), green; APP, blue. (Top right) Close-up view of the hybrid β sheet. The three-stranded β sheet comprises a β strand from APP and two β strands from the extended loop sequence between the NTF and CTF of PS1. (Bottom right) Close-up structural comparison between free APP (orange) and APP bound to γ -secretase (blue). The two initial cleavage sites of γ -secretase are marked by arrows.

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RESEARCH ARTICLE

STRUCTURAL BIOLOGY

Recognition of the amyloid precursor protein by human γ -secretase

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Cleavage of amyloid precursor protein (APP) by the intramembrane protease γ -secretase is linked to Alzheimer's disease (AD). We report an atomic structure of human γ -secretase in complex with a transmembrane (TM) APP fragment at 2.6-angstrom resolution. The TM helix of APP closely interacts with five surrounding TMs of PS1 (the catalytic subunit of γ -secretase). A hybrid β sheet, which is formed by a β strand from APP and two β strands from PS1, guides γ -secretase to the scissile peptide bond of APP between its TM and β strand. Residues at the interface between PS1 and APP are heavily targeted by recurring mutations from AD patients. This structure, together with that of γ -secretase bound to Notch, reveal contrasting features of substrate binding, which may be applied toward the design of substrate-specific inhibitors.

The hallmark of Alzheimer's disease (AD) is the presence of amyloid plaques in the brain of AD patients (1, 2). The primary components of the amyloid plaque are β -amyloid peptides (A β s) derived from the amyloid precursor protein (APP) (3). The type I transmembrane (TM) protein APP is first cleaved by α - or β -secretase to generate a transmembrane fragment of 83 or 99 residues (APP-C83 or APP-C99), respectively (4, 5) (fig. S1A). APP-C99 is then cleaved by γ -secretase through its endopeptidase activity to generate the 48-residue peptide A β 48 or the 49-residue peptide A β 49 (6–8). Subsequent cleavages of A β 49 by the C-terminal peptidase activity of γ -secretase results in the sequential generation of A β 46, A β 43, and A β 40 (3, 9) (fig. S1A). Similarly, cleavages of A β 48 lead to the production of A β 45, A β 42, and A β 38. Of these, A β 42 and A β 43 are particularly prone to aggregation and formation of amyloid plaques (3, 10, 11). In addition to APP, the Notch receptor is also a substrate of α - and γ -secretases (12). After cleavage by α -secretase, the resulting transmembrane Notch fragment is cleaved by γ -secretase to generate an intracellular signaling domain (13). A model of stepwise substrate binding by γ -secretase was purposed on the basis of systematic photoaffinity mapping (14).

Human γ -secretase comprises four subunits: presenilin (PS), PEN-2, APH-1, and nicastrin (NCT) (15, 16). As the catalytic subunit of γ -secretase,

presenilin is an aspartyl protease with two catalytic Asp residues (7) and has two isoforms (PS1 and PS2). During γ -secretase assembly, PS1 undergoes autoproteolysis to produce an N-terminal fragment (NTF) and a C-terminal fragment (CTF) (17). PEN-2 is required for γ -secretase maturation, APH-1 stabilizes the complex (18), and NCT is thought to play a role in substrate binding (19). More than 200 AD-associated mutations have been identified in PS1, most of which result in elevated A β 42/A β 40 ratios (20).

The prevailing amyloid hypothesis postulates that the amyloid oligomers directly contribute to the development of AD (11, 21–23), making inhibition of γ -secretase a potential therapeutic strategy for AD treatment (24–26). Unfortunately, perhaps because they also inhibit Notch cleavage (27), γ -secretase inhibitors cause severe side effects without any clear clinical benefits to AD patients. Here we report the cryo-electron microscopy (cryo-EM) structure of human γ -secretase in complex with a transmembrane APP fragment at 2.6-Å resolution. A hybrid β sheet between PS1 and the substrate is essential for the proteolytic activity of γ -secretase. Comparison of this structure with that of the γ -secretase–Notch complex (28) reveals distinctive features that may be exploited for development of substrate-specific inhibitors. Notably, the residues at the interface between PS1 and APP are heavily targeted for mutations in early-onset AD patients.

Preparation of a γ -secretase–APP complex

The γ -secretase–APP complex is transient and has long defied all efforts at isolation. We developed a chemical cross-linking strategy that aims to stabilize the transient γ -secretase–substrate complex. Using this approach, we obtained a cross-linked complex between γ -secretase (PS1–NTF–Q112C and CTF–D385A) and a 100-residue Notch fragment (Notch-100, P1728C) and determined its cryo-EM structure (28). The

catalytic mutation D385A (Asp³⁸⁵→Ala) in PS1 is required to prevent substrate cleavage by γ -secretase, and the NTF and CTF were coexpressed to mimic the outcome of PS1 autoproteolysis. On the basis of sequence alignment (fig. S1B), APP-C83 corresponds to Notch-100, with Val⁶⁹⁵ of APP corresponding to Pro¹⁷²⁸ of Notch.

We applied the same strategy to generate four APP-C83 mutants, each with a cysteine substitution in a four-residue stretch, and individually examined their cross-linking efficiency with PS1 (NTF–Q112C, CTF–D385A) in γ -secretase (fig. S1C). Only APP-C83 (V695C) was completely cross-linked to PS1. Formation of a stable complex between γ -secretase (PS1–Q112C/D385A) and APP-C83 (V695C) strictly depended on cross-linking in the absence of the reducing agent dithiothreitol (DTT) (fig. S1D). Notably, the mutation V695C allowed retention of APP-C83 cleavage by γ -secretase (fig. S1E). This strategy allowed purification of a large amount of human γ -secretase (PS1–Q112C/D385A, PEN-2, APH-1aL, and NCT) cross-linked to its substrate APP-C83 (V695C) (Fig. 1A and fig. S1F). The disulfide bond in the purified complex could be reduced by DTT (Fig. 1A).

Structure of the γ -secretase–APP complex

We analyzed the cross-linked human γ -secretase–APP-C83 complex by single-particle cryo-EM and determined its structure at an average resolution of 2.6 Å (Fig. 1B, figs. S2 to S5, and table S1). In final atomic model, 34 residues from APP constitute two fragments: one spanning residues 688 to 693 and another spanning residues 699 to 726. An intervening five-residue stretch (residues 694 to 698) that includes the cross-linking site V695C is disordered in the EM density map (Fig. 1C and fig. S6, A and B). Unlike Notch, the APP TM fragment contains few bulky amino acids and no aromatic residues (fig. S1, A and B). Nonetheless, the side-chain assignment of the APP fragment spanning residues 699 to 726 was unambiguously assisted by four hydrophobic residues: Met⁷⁰⁶, Ile⁷¹⁸, Met⁷²², and Leu⁷²³. Similar to Notch (28), the structurally resolved APP sequence comprises an N-terminal loop, a TM helix, and a C-terminal β strand (Fig. 1C).

Compared with that of free APP (29), the TM helix in the γ -secretase–bound APP is unwound by one full helical turn at the C-terminal end (Fig. 1D). Consequently, three residues of the TM helix (Thr⁷¹⁹, Leu⁷²⁰, and Val⁷²¹) in free APP adopt a fully extended conformation upon binding to γ -secretase. This structural change allows cleavage of the peptide bond to occur either between Thr⁷¹⁹ and Leu⁷²⁰, which results in A β 48, or between Leu⁷²⁰ and Val⁷²¹, which yields A β 49 (Fig. 1D). The extended conformation of the residues 718 to 721 is accompanied by a characteristic β strand (Val⁷²² to Lys⁷²⁵) that is present only in the γ -secretase–bound APP, not in free APP.

The APP TM helix, along with the β strand, traverse through a central pore formed by TM2, TM3, TM5, TM6, and TM7 of PS1 (Fig. 2A). TM2 of PS1, implicated in substrate recruitment

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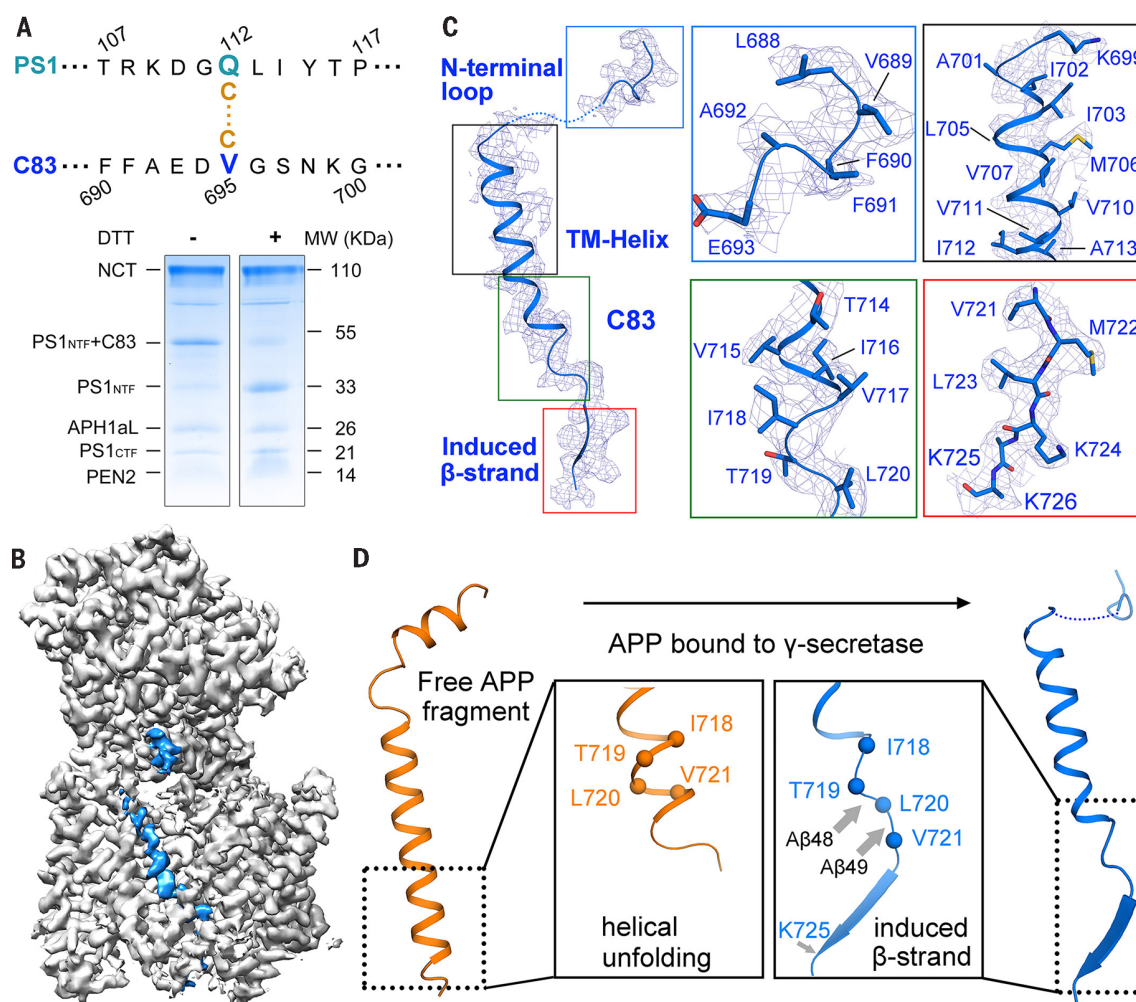


Fig. 1. Cryo-EM structure of human γ -secretase bound to a TM fragment of amyloid precursor protein (APP-C83). (A) The human γ -secretase-APP-C83 complex is stabilized by a disulfide bond between Cys¹¹² of PS1-Q112C and Cys⁶⁹⁵ of APP-V695C. The purified γ -secretase-APP-C83 complex was visualized by SDS-PAGE through Coomassie staining (lower panel). The cross-linked fragment between PS1-NTF and APP-C83, which was formed in the absence of the reducing agent DTT, can be reduced by DTT in vitro, generating free PS1-NTF. For details, see fig. S1 and the Materials and methods section. MW, molecular weight. (B) Overall EM density map of human γ -secretase (gray) in complex with APP-C83 (blue). (C) Structure of APP-C83 from the γ -secretase-APP-C83 complex. The EM density for APP-C83 (left) and close-up views on four segments of APP-C83 (right) are shown. The contour level of the EM

density for the entire APP-C83 is 5σ . The contour levels for the four focused regions are between 5.2σ and 6σ . (D) Structural comparison of the APP TM fragment in its free and γ -secretase-bound states. Compared to the free state (orange) [PDB ID: 2LLM (29)], the N and C termini of the γ -secretase-bound APP fragment (blue) undergoes marked changes. The N-terminal helix is replaced by a coil, and the C-terminal helix unwinds into an extended conformation to expose the potential cleavage sites and form a β strand on the intracellular side. Notably, cleavage after Thr⁷¹⁹ or Leu⁷²⁰ results in A β 48 or A β 49, respectively. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

(30), is dynamic and disordered in substrate-free γ -secretase (31) but becomes ordered upon binding to the substrate and contributes to APP recognition. TM2 of PS1 and the TM of APP-C83 are located on the convex side of the horseshoe-shaped TM domain of γ -secretase (fig. S5, A and B). Most notably, the APP β strand forms an antiparallel three-stranded β sheet with two induced β strands from PS1: β 1 (residues 287 to 290) at the C-terminal end of the structurally resolved portion of NTF and β 2 (377 to 381) at the N-terminal end of CTF (Fig. 2A, inset, and fig. S5C). Formation of the hybrid β sheet is ac-

companied by a rearrangement of the C-terminal portion of TM6 in PS1: The TM6 helix in substrate-free γ -secretase is unraveled with substrate bound and after a rigid loop (residues 263 to 267) continues as a short α helix (designated as TM6a) (Fig. 2B). Notably, similar changes in TM6 are also observed in DAPT-bound γ -secretase (32) (fig. S6C), but formation of the hybrid β sheet is specific to substrate-bound γ -secretase.

These structural changes are stabilized by intramolecular interactions (Fig. 2C). In particular, Arg²⁷⁸ and Glu²⁸⁰, which are located in the loop connecting TM6a and the strand β 1, orchestrate

a network of hydrogen bonds (H-bonds). The carboxylate side chain of Glu²⁸⁰ makes a bifurcated H-bond to the hydroxyl groups of Tyr¹⁵⁴ and Tyr¹⁵⁹, both from TM2 of PS1. These interactions are buttressed by two additional H-bonds from Arg²⁷⁸ to Glu²⁸⁰ and Tyr¹⁵⁹ (Fig. 2C). Consistent with the importance of these interactions, the mutation E280A has been observed in hundreds of early-onset AD patients (33, 34). In addition, Leu²⁷¹ and Thr²⁷⁴ from TM6a make van der Waals contacts to Val¹⁵¹ and Tyr¹⁵⁴. Consistent with the structural observations, Tyr¹⁵⁴, Val²⁷¹, and Thr²⁷⁴ are all targets of AD-associated

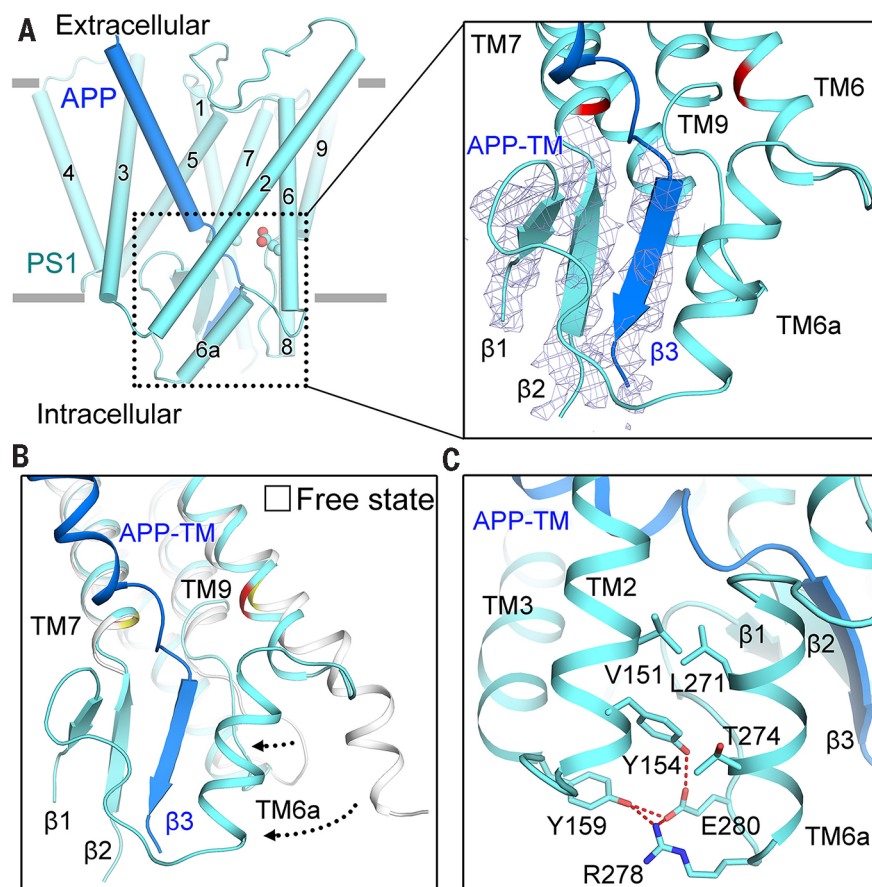


Fig. 2. Structural changes of PS1 and APP-C83 upon association. (A) Overall structure of PS1 bound to APP-C83. The TM of APP-C83 is surrounded by TM2, TM3, TM5, TM6, and TM7 of PS1. In contrast to the substrate-free state (31), TM2 of PS1 can be clearly identified in the substrate-bound state. A hybrid β sheet is formed on the intracellular side, between the β strand from APP and two β strands ($\beta 1$ and $\beta 2$) from loop 2 of PS1. The catalytic residues are shown in red. The EM density of the β sheet is shown at a contour level of 6.5σ . (B) Structural comparison of TM6 between the APP-bound state (cyan) and substrate-free state [gray; PDB ID: 5A63 (31)]. Superimposition of the two structures reveals pronounced translocation of the C-terminal portion of TM6 toward the bound substrate APP-C83. (C) Close-up view of the newly formed helix TM6a. H-bonds are represented by red dashed lines. TM6a interacts with TM2 through a combination of H-bonds and van der Waals contacts. Glu²⁸⁰ appears to anchor this interface by contributing three H-bonds.

mutations, which result in abrogation of APP-C99 cleavage *in vitro* by the corresponding γ -secretase variants (20).

APP recognition by human γ -secretase

The N-terminal half of the APP TM helix is partially exposed to lipid membrane and thus makes sparse interactions with surrounding residues in PS1 (Fig. 3A). Met⁷⁰⁶ and Val⁷⁰⁷ of APP make van der Waals contacts to Tyr²⁴⁰ and Ile¹¹⁴ of PS1, respectively; whereas Thr⁷¹⁴ and Val⁷¹⁵ of APP closely stack against the hydrophobic residues Met¹⁴⁶ and Trp¹⁶⁵. These interactions appear to be brought into registry by a specific H-bond between the carbonyl oxygen of Ile⁷¹² from APP and the hydroxyl group of Ser¹⁶⁹ from TM3 of PS1 (Fig. 3A). The hydroxyl group of Ser¹⁶⁹ is also within H-bond distance of the carbonyl oxygen of Trp¹⁶⁵.

Compared to the N-terminal half, the C-terminal half of the APP TM helix mediates relatively dense

van der Waals contacts (Fig. 3B). Val⁷¹⁷ of APP is nestled in a shallow hydrophobic pocket formed by three PS1 residues: Phe²³⁷, Ile³⁸⁷, and Phe³⁸⁸. Ile⁷¹⁸ of APP closely contacts Met¹⁴⁶, Thr¹⁴⁷, and Leu²⁶⁸ of PS1, whereas Leu⁷²⁰ interacts with Ala⁴³⁴, Leu⁴³⁵, and Gly³⁸⁴. Notably, Ala⁴³⁴ and Leu⁴³⁵ are part of the PAL motif, which has previously been implicated in substrate recognition (35). These interactions are anchored by a H-bond between the carbonyl oxygen of Thr⁷¹⁹ from APP and the amide group of Gly³⁸⁴ from PS1 (Fig. 3B). The mutation G384A in PS1 causes early-onset AD (36); compared with wild-type enzyme, the γ -secretase variant that contains the mutation G384A in PS1 substantially increases the A β 42/A β 40 ratio (20).

The β strand of APP interacts with strand $\beta 2$ and the PAL motif of PS1, mostly through main-chain H-bonds (Fig. 3C). These interactions, together with those involving the C-terminal

half of the APP TM helix, facilitate the extended conformation of the APP residues 718 to 721. The carboxylate of the catalytic residue Asp²⁵⁷ is positioned approximately 6 to 7 Å away from the scissile peptide bond between residues 719 and 720 or 720 and 721 (Fig. 3D). In our study, the other catalytic residue (Asp³⁸⁵) in PS1 was mutated to Ala to prevent cleavage of the bound APP substrate. It is possible that such a mutation might also lead to slight perturbation of the local conformation in the active site.

The TM segment of APP is accommodated in a cut-through channel of PS1 (fig. S7A). The cleavage products of APP-C99 by γ -secretase follow two lines: A β 49-A β 46-A β 43-A β 40 and A β 48-A β 45-A β 42-A β 38. For either line, the C-terminal residues are located on the same side of the TM helix (fig. S7B). Analysis of the binding pockets for the side chains of these residues reveals intriguing features (fig. S7, C to F). For example, the mutation I716F in APP is known to markedly elevate the A β 42/A β 40 ratio (37). In the structure, the binding pocket of Ile⁷¹⁶ is large enough to accommodate the aromatic side chain of Phe (fig. S7E); the mutation I716F may favor production of A β 48 through stabilization of the corresponding APP conformation. In addition, the binding pocket for Leu⁷²⁰-Val⁷²¹-Met⁷²² of APP, which is located next to the γ -secretase cleavage site, appears to follow the large-small-large pattern as previously described (38) (fig. S7, G and H).

To corroborate the structural observations, we generated four γ -secretase mutants, each with a deletion or mutation in PS1, and examined their activity toward the APP-C83 substrate (Fig. 3E). Deletion of $\beta 1$ (residues 288 to 290), $\beta 2$ (residues 377 to 381), or the PAL motif (residues 433 to 435) crippled the proteolytic cleavage of APP-C83. The missense mutation L432P, which presumably affects the local conformation and stability of the APP β strand, also abolished the activity.

Differential recognition of APP and Notch

Structural elucidation of APP recognition by human γ -secretase allows comparison with Notch recognition (28). Although the global conformation of γ -secretase remains unchanged between the APP- and Notch-bound states, substantial structural rearrangements are observed in the substrate-binding regions of PS1 and are likely induced by differential substrate binding (fig. S8A). The C-terminal half of TM2 and the N-terminal half of TM3, along with the short intervening linker sequence, undergo noticeable shift. In addition, the loop sequences preceding TM2 also exhibit different conformations between the APP- and Notch-bound states.

These differences are caused by the distinct sequence features of APP and Notch. Unlike the Notch TM helix that contains four Phe residues and four β -branched residues, the APP helix contains no aromatic residues and 11 β -branched residues (fig. S1, A and B). Consequently, compared with Notch, the APP helix exhibits distinctive surface features and appears slightly smaller. Two distinct sets of amino acids from

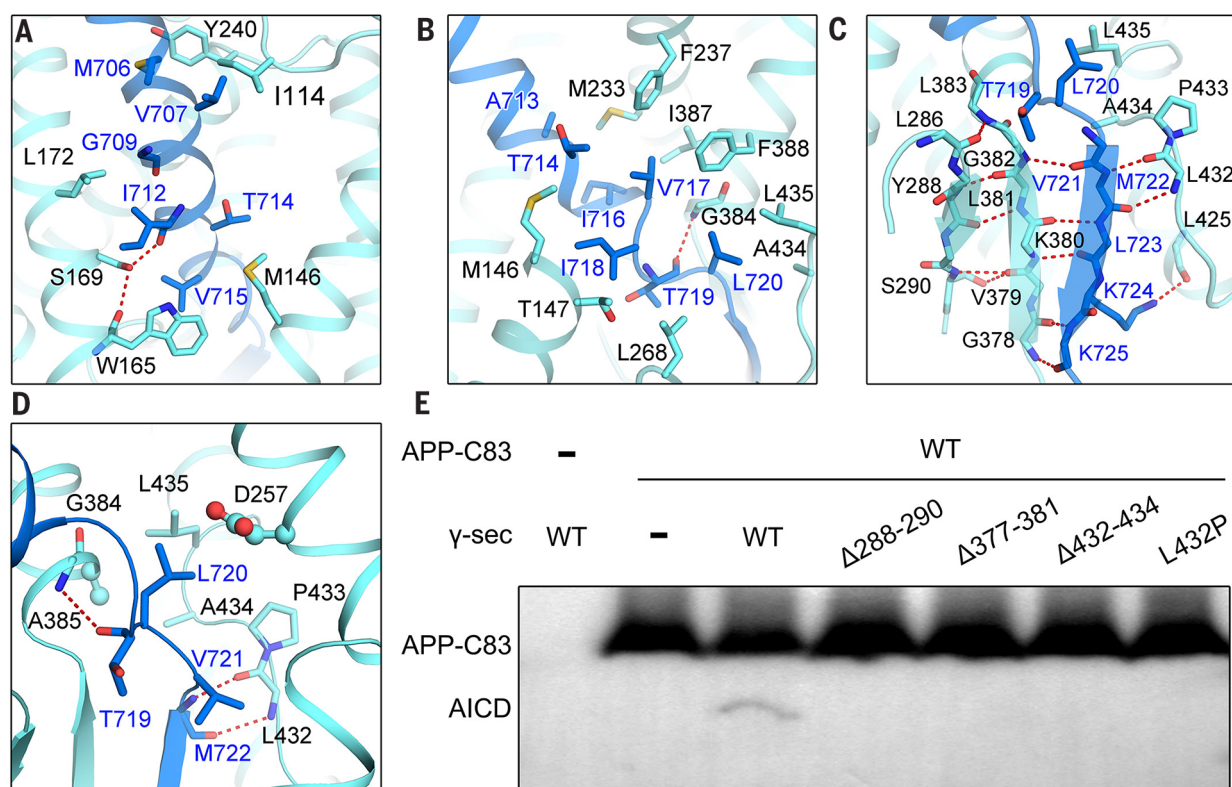


Fig. 3. Recognition of APP-C83 by human γ -secretase. (A) Close-up view of the interactions of the N-terminal half of the TM helix of APP-C83 (blue) with surrounding structural elements from PS1 (cyan). H-bonds are represented by red dashed lines. A conserved H-bond between the side chain of Ser¹⁶⁹ and the carbonyl oxygen of Ile⁷¹² appears to anchor this interface. (B) Close-up view of the interactions of the C-terminal half of the TM helix and the ensuing residues of APP-C83 (blue) with surrounding structural elements from PS1 (cyan). This interface is anchored by a conserved H-bond between the amide group of Gly³⁸⁴ of PS1 and the carbonyl oxygen of Thr⁷¹⁹ of APP-C83. (C) Close-up view of the interactions surrounding the APP β strand. The PAL motif

(Pro⁴³³-Ala⁴³⁴-Leu⁴³⁵) from PS1, which is implicated in substrate binding, stabilizes the APP β strand and orients the scissile peptide bonds. (D) Close-up view of the active site of PS1 and the cleavage site in APP-C83. The active site residues in PS1, Asp²⁵⁷ and Ala³⁸⁵ (replacing Asp³⁸⁵), are displayed in ball-and-stick representation. Cleavage of Thr⁷¹⁹-Leu⁷²⁰ or Leu⁷²⁰-Val⁷²¹ results in A β 48 or A β 49, respectively. (E) Formation of the β sheet is indispensable for γ -secretase cleavage. Deletion of β 1 (residues 288 to 290), β 2 (residues 377 to 381), or the PAL motif (residues 432 to 434) in PS1 leads to abrogation of the cleavage activity of γ -secretase. AICD, APP intracellular domain; WT, wild type.

PS1 are employed to interact with the TM helix from APP and Notch. Leu⁸⁵, Thr¹⁴⁷, and Ile²⁸⁷ contribute to APP but not Notch binding (fig. S8B), whereas Phe¹⁷⁶ and Phe¹⁷⁷, along with eight other PS1 residues, directly interact with residues from Notch but not APP (fig. S8C). A set of residues, exemplified by Ser¹⁶⁹ and Gly³⁸⁴, is involved in binding to both APP and Notch (fig. S8D). The TM helix from both APP and Notch appears to be anchored by a pair of conserved H-bonds donated by the hydroxyl group of Ser¹⁶⁹ and the amide group of Gly³⁸⁴ (fig. S8E). The acceptors of these H-bonds are carbonyl oxygen atoms.

Differences in substrate recognition might be exploited to facilitate the discovery of substrate-specific inhibitors of γ -secretase. For both substrates, the N-terminal half of the TM helix is exposed to lipid membrane (Fig. 4A), with five consecutive bulky residues (LHFMY) in Notch (fig. S1A). Because these bulky residues in Notch occupy more space than the corresponding residues in APP, an inhibitor could target this region

of PS1 to selectively block Notch but not APP binding. The C-terminal half of the TM helix shows alternating size differentials between APP and Notch. Along the helical axis, the Val⁷¹⁵-Ile⁷¹⁶ segment of APP is smaller than the Phe¹⁷⁴⁸-Phe¹⁷⁴⁹ segment in Notch (Fig. 4B). In contrast, the Ile⁷¹⁸-Thr⁷¹⁹-Leu⁷²⁰ segment of APP is considerably bulkier than the Gly¹⁷⁵¹-Cys¹⁷⁵²-Gly¹⁷⁵³ segment of Notch (Fig. 4C). It is conceivable that a small inhibitor bound to this region of PS1 may selectively inhibit the cleavage of APP but not Notch. Notably, this latter segment encompasses the scissile peptide bond that generates A β 48. Systematic comparison of the surface features between the APP and Notch TM fragments reveals candidate binding sites for such an inhibitor (Fig. 4D).

AD-associated mutations at the PS1-APP interface

Structure determination of the human γ -secretase-APP complex allows assessment of the impact of AD-associated mutations on the interactions

between γ -secretase and APP. On one hand, 247 AD-associated mutations in PS1 have been reported to affect 136 amino acids (www.alzforum.org/mutations/). Of these 136 residues, 59 are targeted for mutations to two or more types of amino acids (recurring mutations). One hundred twenty-five of the 136 affected residues, or 92% of the total, and the vast majority of the 59 mutational hotspot residues can be mapped onto the structurally resolved regions of PS1 (Fig. 5A). On the other hand, 30 AD-associated mutations in APP have been identified to affect 17 residues, of which 11 (65% of the total) can be seen in our structure. Among the 11 identified residues, two reside in the extracellular loop and nine are located in the TM helix and β strand (Fig. 5, B and C).

The 59 mutational hotspot PS1 residues can be classified into four distinct groups. The first group of six residues is located in the extended sequences on the extracellular side preceding TM2 (loop-1) (Fig. 5A). These residues likely play a role in substrate recruitment and delivery into

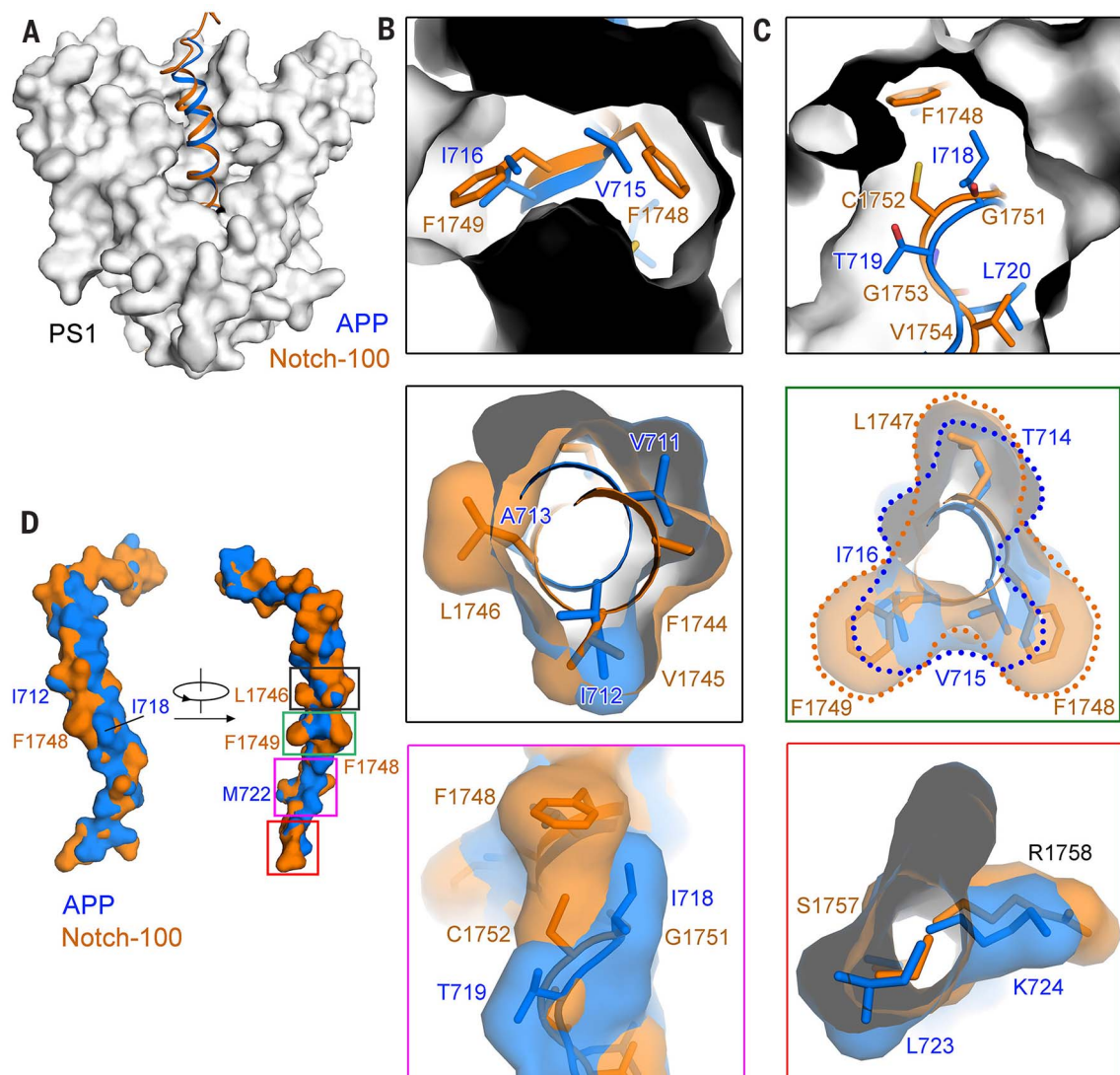


Fig. 4. Differential recognition of Notch and APP by human γ -secretase.

(A) Overall comparison of APP (marine) and Notch (orange) bound to PS1 (gray). PS1 is shown in surface representation. (B) Close-up view of the binding site of PS1 for the Val⁷¹⁵-Ile⁷¹⁶ segment of APP or the Phe¹⁷⁴⁸-Phe¹⁷⁴⁹ segment of Notch. The APP segment is considerably smaller than that of Notch. (C) Close-up view of the binding site of PS1 for the Ile⁷¹⁸-Thr⁷¹⁹-Leu⁷²⁰

segment of APP or the Gly¹⁷⁵¹-Cys¹⁷⁵²-Gly¹⁷⁵³ segment of Notch. The APP segment is considerably larger than that of Notch. (D) Systematic analysis of the side-chain features of the APP-TM and Notch-TM fragments. Shown at left are two views of the superimposition of the APP-TM (blue) and Notch-TM (orange) fragments in their surface representation. The four boxed segments are depicted in the middle and right panels. Bulky residues are labeled.

the active site (Fig. 5D). The second group of 19 residues has side chains pointing into the substrate-binding pore (Fig. 5D). The third group of 17 residues is located in the region that undergoes marked conformational changes upon substrate binding (i.e., TM6a, TM2, and the N-terminal portion of TM3) (Fig. 5E). Mutations in the second and third groups are likely to alter interactions with the substrate and/or hinder conformational changes induced by substrate binding. The fourth group—the remainder of the 59 hotspot residues—does not belong to any of the above three groups. But mutation of any of these residues, exemplified by Gly²⁰⁶ and Gly²⁰⁹, may affect residues of the other three groups. For example, mutation of Gly²⁰⁶ or Gly²⁰⁹ likely affects the conformation of the adjacent residue

Leu¹⁷⁴, which may propagate to Ser¹⁶⁹ or Phe¹⁷⁷, both in the second group (Fig. 5F).

Discussion

The structure reported here reflects that of a mutated γ -secretase cross-linked to a mutated substrate. As such, the structure may represent a snapshot, and other structural variations of the γ -secretase-substrate complex are possible. Nevertheless, the atomic structure of the human γ -secretase-APP-C83 complex provides a physical basis for understanding the consequences of AD-associated mutations. The APP residues targeted for mutation in AD patients are clustered in the C-terminal half of the APP TM helix and the β strand, which are sequentially positioned before and after the scissile peptide bonds, respectively

(Fig. 5A). Notably, the vast majority of the mutational hotspot PS1 residues appear to directly or indirectly affect APP binding and cleavage. In particular, a sizable fraction of these residues are clustered in the regions surrounding the C-terminal half of the APP TM helix and the β strand (Fig. 5, B and C). This analysis implicates a role of APP recruitment and cleavage in the pathogenesis of AD.

Despite the differences in substrate recognition, design of substrate-specific inhibitors of γ -secretase is challenging for two reasons. First, APP and Notch, each comprising a TM helix and a β strand, bind to the same general location in γ -secretase. Second, the recognition mechanism between APP and Notch shares two common themes. In both cases, the TM helix is anchored

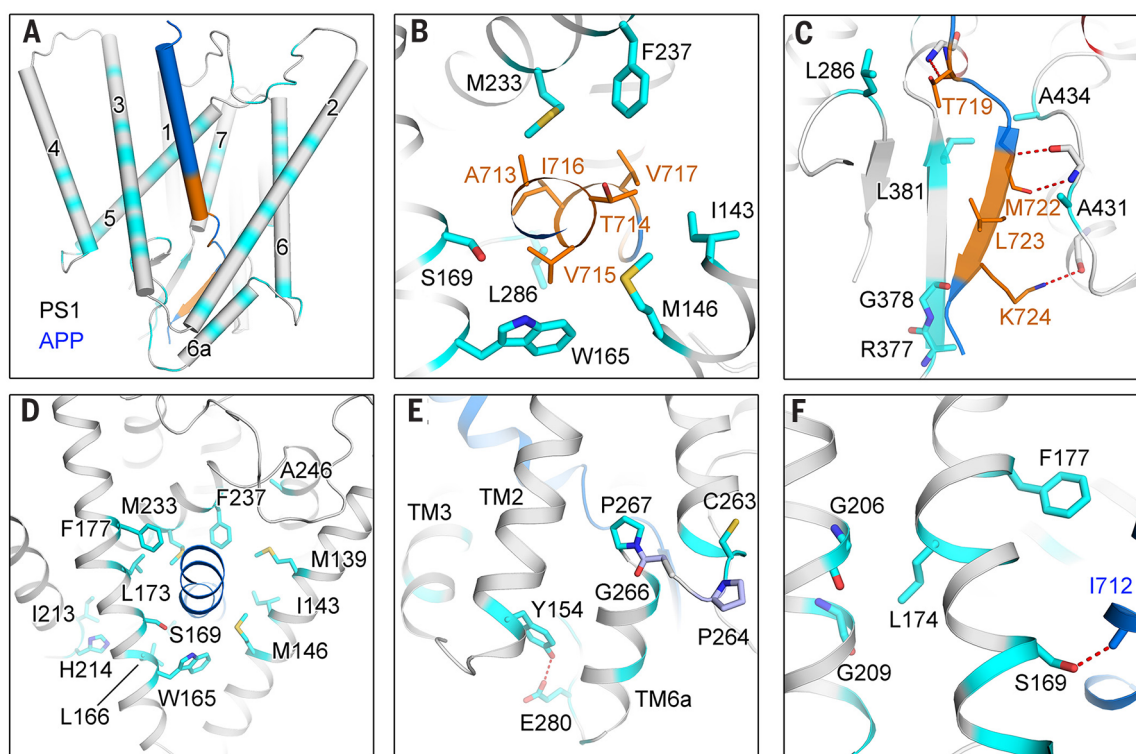


Fig. 5. PS1 residues involved in the recruitment and cleavage of APP are predominantly targeted for recurring mutations in AD patients.

(A) Mapping of AD-associated mutations onto the structure of human γ -secretase bound to APP-C83. AD-associated mutations in APP are shown in orange. AD-associated recurring mutations in PS1 are highlighted in cyan (20). Most recurring mutations are located along the binding pore of APP-TM. (B) Close-up view of the interface between the C-terminal half of the APP TM helix and surrounding PS1 elements. All residues shown in the stick model are targeted for recurring mutations in AD patients. (C) Close-up view of the interface between the β strand of APP

and the surrounding elements of PS1. All residues shown in the stick model are targeted for recurring mutations in AD patients. (D) 19 residues in PS1 that are targeted for recurring mutations in AD patients place their side chains toward the interior of the substrate-binding pore. (E) A close-up view on the residues that appear to stabilize the structural arrangement upon APP binding. These residues are targeted for recurring mutations in AD patients. (F) A close-up view on the PS1 residues Leu¹⁷⁴, Gly²⁰⁶, and Gly²⁰⁹. These three residues are subject to recurring mutations in AD patients. Each mutation likely affects the local conformation, which propagates to those residues that directly contribute to substrate binding.

through a pair of conserved H-bonds, and substrate cleavage is oriented through formation of a hybrid β sheet between the substrate and PS1. Nonetheless, careful analysis reveals small but important structural differences between APP and Notch, especially in the regions toward the C-terminal end of the TM helix (Fig. 4). An inhibitor that selectively targets APP cleavage is likely to be bimodal. One end of the inhibitor should ideally bind to the surface specific to APP-bound γ -secretase, which, for example, may involve the N-terminal portion of TM3. The other end of the inhibitor, presumably small in size, may be fitted into the space that would be occupied only by the bulkier residues of APP, which, for instance, could be the region surrounding Ile⁷¹⁸-Thr⁷¹⁹-Leu⁷²⁰.

APP-C99, but not APP-C83, is the precursor to the aggregating β -amyloid peptides. In this study, APP-C83, rather than APP-C99, was chosen to be the substrate of γ -secretase. This choice of substrate is based on sequence alignment between APP-C99 and Notch-100 as well as the fact that γ -secretase interacts with APP-C83 more strongly than with APP-C99 (39). Consequently, the endopeptidase cleavage of APP-C99 by γ -secretase is less efficient com-

pared with that of APP-C83 (fig. S9, A and B). However, the observed interactions between APP-C83 and PS1 are likely to be identical to those in the γ -secretase-APP-C99 complex. Compared to APP-C83, the extra 16 amino acids at the N-terminus of APP-C99 may play an additional regulatory role in formation of the γ -secretase-substrate complex and possibly catalysis (14, 40).

Compared to free γ -secretase, the overall structures of the other three subunits (PEN-2, APH-1, and NCT) of APP-bound γ -secretase remain largely unchanged (fig. S6, D to H). The putative substrate-binding residue Glu³³³ and its surrounding structural elements also show few changes between the substrate-free and APP-bound states of γ -secretase (fig. S6E). In our structure, the N-terminal two residues Leu⁶⁸⁸ and Val⁶⁸⁹ of APP-C83 directly interact with surrounding residues from NCT (fig. S9C). It is possible that residues on the N-terminal side to Lys⁶⁸⁷ may modulate this interaction. Consistent with this analysis, in the absence of DTT, γ -secretase forms a stable complex with APP-C83 but not with APP-C99 (fig. S9D).

The structure of human γ -secretase bound to the amyloid precursor protein lends strong support to the helix-unwinding model of suc-

cessive substrate cleavage by γ -secretase (20). More importantly, this structure allows comparison of APP and Notch recognition by γ -secretase and rationalization of AD-associated mutations. As such, this structure serves as an important framework for discovery of substrate-specific inhibitors of γ -secretase and for understanding the biological functions of γ -secretase as well as the disease mechanisms of AD.

Materials and methods

Clones and plasmids

The coding DNA sequences for human PS1 and its variants, APH-1aL, PEN-2, NCT, and the APP fragments were individually cloned into the pMLink vector as previously described (41). All plasmids used for transfection of mammalian cell were prepared using the EndoFree Plasmid Maxi Kit (Cwbiotech).

Rationale and design of a γ -secretase-APP complex

To obtain a stable γ -secretase-C83 complex, we sought to stabilize the enzyme-substrate complex through an engineered disulfide bond as reported in the case of the γ -secretase-Notch complex (28). The APP sequences comprise a large

extracellular domain, a TM region, and a short intracellular domain known as AICD (fig. S1A). On the basis of sequence alignment, we chose a four-residue stretch of APP-C83 that corresponds to the region of exhaustive Cys mutation in Notch (fig. S1B). Because PS1 undergoes auto-proteolysis during γ -secretase assembly to produce an NTF and a CTF, the NTF and CTF of PS1 were coexpressed together with the other three subunits (PEN-2, APH-1aL, and NCT) to generate recombinant γ -secretase. The catalytic residue Asp³⁸⁵ was mutated to Ala in PS1 to avoid substrate cleavage. Then we generated four APP-C83 mutants, each with a cysteine substitution in the four-residue stretch, and individually examined their cross-linking efficiency with PS1 (NTF-Q112C, CTF-D385A) in γ -secretase (fig. S1C). Only APP-C83 (V695C) was completely cross-linked to PS1. Formation of a stable complex between γ -secretase (PS1-Q112C/D385A) and APP-C83 (V695C) strictly depended on cross-linking in the absence of the reducing agent DTT (fig. S1D). Importantly, the mutation V695C allowed retention of APP-C83 cleavage by the wild-type γ -secretase (fig. S1E). Nonetheless, the cleavage activity is reduced compared to the wild-type APP-C83. This strategy allowed purification of a large amount of human γ -secretase (PS1-Q112C/D385A, PEN-2, APH-1aL, and NCT) cross-linked to its substrate APP-C83 (V695C) (Fig. 1A and fig. S1F).

Protein expression and purification

Human γ -secretase (PS1-NTF-Q112C, PS1-CTF-D385A, PEN-2, APH-1aL, NCT) and APP-C83 (residues 688–771, V695C) were coexpressed in HEK293 cells and purified as previously described (28). Cell membranes were resuspended in the lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl) supplemented with 0.1% (w/v) digitonin and 1% (w/v) CHAPSO. PEN-2 has an N-terminal FLAG tag for affinity purification. APP-C83 is tagged with Myc and 6xHis at its C terminus. The γ -secretase-APP-C83 complex eluted from the affinity column was further purified by gel filtration (Superose-6, GE Healthcare) in the lysis buffer supplemented with 0.1% digitonin.

For each of the γ -secretase variants used in the proteolytic activity assay, PS1 carries a specific missense mutation or deletion. Purification of such γ -secretase variants was performed as previously described (31). PS1 was detected by an anti-PS1 monoclonal antibody (Merck), and the Myc tag was detected by an anti-Myc monoclonal antibody (Cwbiotech, Beijing).

Electron microscopy

Cryo-EM samples were prepared as described (31). 4- μ l aliquots of recombinant human γ -secretase cross-linked to APP-C83 were applied to glow-discharged holey carbon grids (Quantifoil Au R1.2/1.3, 300 mesh). The grids were blotted for 3 s and flash frozen in liquid ethane using Vitrobot Mark IV (FEI). The sample was imaged on an FEI Titan Krios transmission electron microscope equipped with a Cs corrector, operating at 300 kV with a nominal magnification of

105,000 $\times$. Images were recorded by a Gatan K2 Summit direct electron detector and a Gatan GIF Quantum energy filter (slit width: 20 eV) using the super-resolution mode. Defocus values varied from -1.0 to -2.2 μ m. Each image was dose fractionated to 32 frames with a total electron dose of ~ 50 e⁻/Å² and a total exposure time of 5.6 s. AutoEMation II (developed by Jianlin Lei) (42) was used for automated data collection. All stacks were motion corrected using MotionCor2 (43) with the binning factor of 2, resulting in a pixel size of 1.091 Å. The defocus values were estimated using Gctf (44) and dose weighing was performed concurrently (45).

Cryo-EM image processing

In total, 6838 movie stacks were recorded (fig. S2). After motion correction and CTF estimation, 6360 micrographs were selected. 3,575,237 particles were autopicked from these 6360 movie stacks using RELION-2.0 (46–49). After two-dimensional (2D) classification, 2,925,279 particles were selected and subjected to 3D classification. The γ -secretase EM map (EMD-3061) was low-pass filtered to 20 Å to generate an initial model (31). The selected particles were subjected to 25 iterations of global angular search 3D classification. Each of the 25 iterations has one class and a step size of 7.5°. For the last iteration (iteration 25) of the global search, the local angular search 3D classification was executed with a class number of five, a step size of 3.75°, and a local search range of 15°. The resulted classes of local search were used to generate multireferences.

For the last six iterations (iterations 20 to 25) of the global search, multireference 3D classification was performed, each with a class number of five, a step size of 3.75°, and a local search range of 15° for 10 iterations. For the last five iterations of the multireference classification, particles from the “good” classes (i.e., those with additional EM density in PS1) were merged and duplicated particles were removed. After the multireference 3D classification, 1,076,837 particles (36.8% of all selected particles after 2D classification) were subjected to another round of multireference 3D classification. Particles from good classes were applied to autorefinement, resulting in a 3.0-Å map. The box size was then changed from 200 pixels to 320 pixels, after which 3D autorefinement improved the resolution to 2.6 Å on the basis of the Fourier shell correlation (FSC) 0.143 criterion (50). The FSC curves were corrected for the effects of a soft mask using high-resolution noise substitution (51). Local resolution variations were estimated using RELION-2.0 (46).

Model building and structure refinement

The initial model used for the γ -secretase-APP-C83 complex was the substrate-free γ -secretase (31). The structure was first refined in real space using PHENIX with secondary structure and geometry restraints (52). APP-C83 was built de novo from a poly-Ala model. The atomic model was manually improved using COOT (53). Sequence assignment was guided by relatively

bulky residues such as Met and Ile. Sixteen glycosylation sites were identified on the basis of clear features in the EM density map. Three cholesterol and two phosphatidylcholines were found to surround the TM domain of γ -secretase. Several EM density lobes resemble phospholipids, but the quality of these densities was insufficient for their assignment. The final atomic model was refined in real space using PHENIX (54). The final atomic model was evaluated using MolProbity (55).

γ -secretase proteolytic activity assay

APP-C83 or APP-C99 with a C-terminal Myc-His₆ tag was overexpressed in *Escherichia coli* and purified using a Ni²⁺-NTA column. The eluted materials were then applied to gel filtration (Superdex-200, GE Healthcare) in the lysis buffer supplemented with 0.5% (w/v) CHAPSO. Purified substrate was mixed with purified γ -secretase in the lysis buffer supplemented with 0.2% (w/v) CHAPSO, 0.1% (w/v) phosphatidylcholine, 0.025% (w/v) phosphatidylethanolamine, and 0.00625% (w/v) cholesterol. The reaction was allowed to proceed at 37°C for 4 hours. The concentrations of all γ -secretase variants were determined using the Bradford method and confirmed by applying aliquots of the variants to SDS-polyacrylamide gel electrophoresis (PAGE). The final γ -secretase concentration was 60 nM in the cleavage assays. The final substrate concentration in the cleavage assay is ~ 8 μ M. The cleavage product AICD-Myc was detected by an anti-Myc monoclonal antibody (Cwbiotech, Beijing).

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S9
Table S1

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RESEARCH ARTICLE SUMMARY

TOXINS

The human gut bacterial genotoxin colibactin alkylates DNA

Matthew R. Wilson*, Yindi Jiang*, Peter W. Villalta, Alessia Stornetta, Paul D. Boudreau, Andrea Carrá, Caitlin A. Brennan, Eunyoung Chun, Lizzie Ngo, Leona D. Samson, Bevin P. Engelward, Wendy S. Garrett, Silvia Balbo†, Emily P. Balskus†

INTRODUCTION: Members of the human gut microbiota have been implicated in the development and progression of colorectal cancer (CRC). These CRC-associated microorganisms may influence carcinogenesis through a variety of mechanisms, including the production of genotoxins. Colibactin is a genotoxic secondary metabolite made by organisms harboring the *pks* genomic island, including certain gut commensal *Escherichia coli* strains (*pks*⁺ *E. coli*). Transient infection of mammalian cells with *pks*⁺ *E. coli* causes cell cycle arrest, DNA double-strand breaks, and senescence. Moreover, colibactin-producing *E. coli* accelerate tumor progression in multiple mouse models of colitis-associated CRC and are overrepresented in patients with familial adenomatous polyposis and CRC. Despite colibactin's strong links to cancer, the active genotoxic metabolite has eluded all isolation attempts, limiting our mechanistic understanding of this association.

RATIONALE: Over the past decade, multiple complementary approaches have provided indirect information about colibactin's chemical structure. Interestingly, the isolation and structural characterization of metabolites from mutant strains of *pks*⁺ *E. coli* revealed that colibactin likely contains a cyclopropane ring, a reactive structural motif found in DNA alkylating natural products. This led us and others to hypothesize that colibactin may covalently modify DNA. To obtain information about the active genotoxin's chemical structure and its mode of action, we sought to identify and structurally characterize colibactin-DNA adducts from human cells infected with *pks*⁺ *E. coli*.

RESULTS: Using untargeted liquid chromatography-mass spectrometry-based DNA adductomics, we compared the DNA adducts present in mammalian cell lines transiently infected

with either *pks*⁺ *E. coli* or a mutant strain missing the *pks* genes. We discovered two adenine adducts that were specific to the cells exposed to *pks*⁺ *E. coli*. These adducts were confirmed to be *pks*-associated by feeding isotopic labeled versions of known colibactin biosynthetic precursors to the *E. coli*-mammalian cell system. The *pks*-dependent adducts were also found in human cells exposed to clinical colibactin-producing *E. coli* isolates and in the colonic

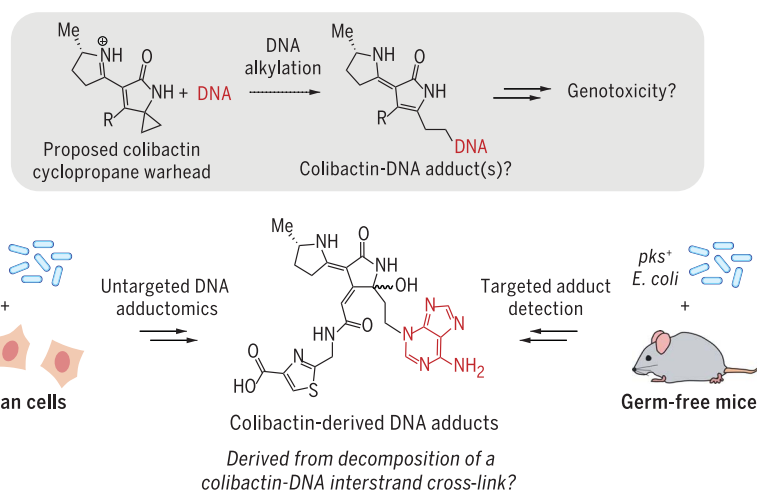
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epithelial cells of mice monocolonized with *pks*⁺ *E. coli*. Chemical synthesis and in vitro DNA alkylation reactions enabled the preparation of an authentic standard

of the adducts. Structural characterization revealed a mixture of two diastereomeric adducts that both contain a 5-hydroxypyrrolidin-2-one ring system with an attached N3-substituted adenine ring. These DNA adducts are generated from ring opening of a reactive, cyclopropane-containing electrophilic warhead, confirming the importance of this structural feature for colibactin's in vivo activity. Because these adducts are too small to derive from the final colibactin structure, we hypothesize that they arise from decomposition of a larger, unstable colibactin-DNA interstrand cross-link. Using a CometChip assay, we detected interstrand cross-link formation in cells infected with *pks*⁺ *E. coli* at the same time point at which we identified the characterized DNA adducts, supporting this proposal.

CONCLUSION: Our results provide direct evidence that the gut bacterial genotoxin colibactin alkylates DNA in vivo, providing mechanistic insights into how colibactin may contribute to CRC. The ability of *pks*⁺ *E. coli* to generate DNA adducts in mammalian cells and in mice strengthens support for the involvement of colibactin in cancer development or progression. Bulky DNA adducts, especially interstrand cross-links, are often cytotoxic and can lead to mutations if not accurately repaired. Colibactin-mediated DNA damage and the ensuing genomic instability could thus potentially be an underlying mediator of colorectal carcinogenesis. The colibactin-derived DNA adducts we identified could serve as a biomarker of *pks*⁺ *E. coli* exposure and will ultimately help to address the question of whether DNA damage inflicted by colibactin-producing gut bacteria contributes to CRC development and progression in humans. ■



Gut commensal *E. coli* strains associated with CRC produce a DNA-alkylating genotoxin.

(Top) The cyclopropane ring found in *pks*-dependent metabolites led us to hypothesize that colibactin alkylates DNA. Me, methyl. (Bottom) Untargeted DNA adductomics revealed colibactin-derived DNA adducts in human cells exposed to colibactin-producing *E. coli*. These adducts also form in mice colonized with *pks*⁺ *E. coli*, confirming that colibactin alkylates DNA in vivo and strengthening its link to cancer.

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RESEARCH ARTICLE

TOXINS

The human gut bacterial genotoxin colibactin alkylates DNA

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Certain *Escherichia coli* strains residing in the human gut produce colibactin, a small-molecule genotoxin implicated in colorectal cancer pathogenesis. However, colibactin's chemical structure and the molecular mechanism underlying its genotoxic effects have remained unknown for more than a decade. Here we combine an untargeted DNA adductomics approach with chemical synthesis to identify and characterize a covalent DNA modification from human cell lines treated with colibactin-producing *E. coli*. Our data establish that colibactin alkylates DNA with an unusual electrophilic cyclopropane. We show that this metabolite is formed in mice colonized by colibactin-producing *E. coli* and is likely derived from an initially formed, unstable colibactin-DNA adduct. Our findings reveal a potential biomarker for colibactin exposure and provide mechanistic insights into how a gut microbe may contribute to colorectal carcinogenesis.

The human gut harbors trillions of microorganisms capable of producing small molecules that mediate microbe-host interactions (1). For example, certain gut commensal and extraintestinal pathogenic strains of *Escherichia coli* and other Proteobacteria produce colibactin, a genotoxin of unknown structure implicated in colorectal cancer pathogenesis. These organisms harbor a 54-kb biosynthetic gene cluster that encodes a non-ribosomal peptide synthetase–polyketide synthase (NRPS-PKS) assembly line (*pks* island), which has been implicated in colibactin biosynthesis (Fig. 1A) (2). *E. coli* containing the *pks* island (*pks*⁺ *E. coli*) cause DNA double-strand breaks (DSBs) in human cell lines and in animals (3), accelerate colon tumor growth under conditions of host inflammation (4, 5), and are found with increased frequency in inflammatory bowel disease, familial adenomatous polyposis, and colorectal cancer patients (6–8). Despite these intriguing links to human disease, our understanding of colibactin's chemical structure

and biological activity is limited because this natural product has eluded isolation.

Colibactin has been exceptionally challenging to isolate and structurally characterize. For example, colibactin's genotoxic activity is contact-dependent and not observed when cells are treated with *pks*⁺ *E. coli* culture supernatants or cell lysates (2). It is also currently unknown how colibactin is transported into mammalian cells. Attempts to directly identify colibactin using comparative metabolite analyses have been unsuccessful, indicating that the active genotoxin may be unstable and/or recalcitrant to isolation. To gain information about colibactin's structure, we and others have isolated and characterized nongenotoxic, *pks*-associated metabolites (9–15) from mutant strains of *pks*⁺ *E. coli* missing a critical peptidase enzyme (CibP), which removes an *N*-myristoyl-D-asparagine “prodrug motif” from a late-stage biosynthetic precursor and is required for genotoxicity (16–18) (Fig. 1B). These metabolites, termed “precolibactins,” are unlikely to be precursors to the mature colibactin because their synthesis requires only a subset of the biosynthetic machinery known to be essential for genotoxic activity. Notably, several precolibactins contain a cyclopropane ring, a structural feature found in DNA alkylating natural products, such as the illudins (19) and duocarmycins (20) (Fig. 1C).

DNA alkylating agents act as electrophiles toward DNA bases, forming covalent modifications known as DNA adducts (21). The discovery of cyclopropane-containing precolibactins has led to the hypothesis that colibactin's mode of action involves DNA alkylation, but there is limited direct evidence to support this idea (10, 11). Reacting a cyclopropane-containing precolibac-

tin with linearized plasmid DNA revealed small amounts of a putative higher-molecular weight adduct by gel electrophoresis, leading to an initial proposal that colibactin cross-links DNA (10). Recent in vitro work using synthetic “colibactin mimics,” compounds designed based on partial biosynthetic information, showed that the cyclopropane ring in a putative CibP cleavage product can be attacked by a thiol nucleophile and is necessary for these molecules to shear purified DNA (22). When artificially dimerized, these colibactin mimics appear to cross-link DNA as assessed by gel electrophoresis (22). *pks*⁺ *E. coli* lacking both the nucleotide excision repair protein UvrB (23) and a self-resistance protein encoded in the *pks* island (CibS) exhibit severe autotoxicity and impaired growth (24), providing indirect support for DNA alkylation and repair of the resulting lesions in colibactin-producing *E. coli* strains. CibS can hydrolyze the cyclopropane ring of a synthetic colibactin mimic, further implicating this functional group in colibactin's activity (25). However, experimental proof that colibactin itself alkylates DNA remains elusive, because colibactin-DNA adducts have not been structurally characterized or identified in biologically relevant settings.

Untargeted DNA adductomics can identify unknown DNA adducts

Owing to the challenges associated with isolating the active genotoxin from *E. coli*, we instead sought to identify the in vivo product(s) of colibactin-mediated DNA damage. We hypothesized that detecting and characterizing colibactin-DNA adducts generated in human cells treated with *pks*⁺ *E. coli* would yield direct information about the active genotoxin's chemical structure and the molecular basis for its DNA damaging activity in a biologically relevant setting. Although targeted liquid chromatography–mass spectrometry (LC-MS)–based methodologies exist to identify previously characterized DNA adducts in cells, detecting unknown DNA adducts represents a considerable challenge because of the low abundance of these modifications and the extraneous false-positive ion signals that derive from the complex matrices of biological samples (26). Indeed, preliminary attempts to identify colibactin-DNA adducts using standard comparative metabolite profiling approaches failed to reveal differences in hydrolyzed DNA samples from HeLa cells treated with either *E. coli* BW25113 pBeloBAC (*pks*[−]) or BAC*pks* (*pks*⁺) strains. To overcome this difficulty, we envisioned exploiting a newly developed, untargeted MS-based DNA adductomics approach (26) to identify colibactin-DNA adducts in cells exposed to *pks*⁺ *E. coli*.

LC-MS³ DNA adductomics identifies adducts in hydrolyzed DNA samples using high-resolution accurate-mass data-dependent constant neutral-loss monitoring of 2'-deoxyribose [116.0474 unified atomic mass unit (u)] or one of the four DNA bases (guanine, 151.0494 u; adenine, 135.0545 u; thymine, 126.0429 u; and cytosine, 111.0433 u) (Fig. 2A) (27). Accurate mass measurement of

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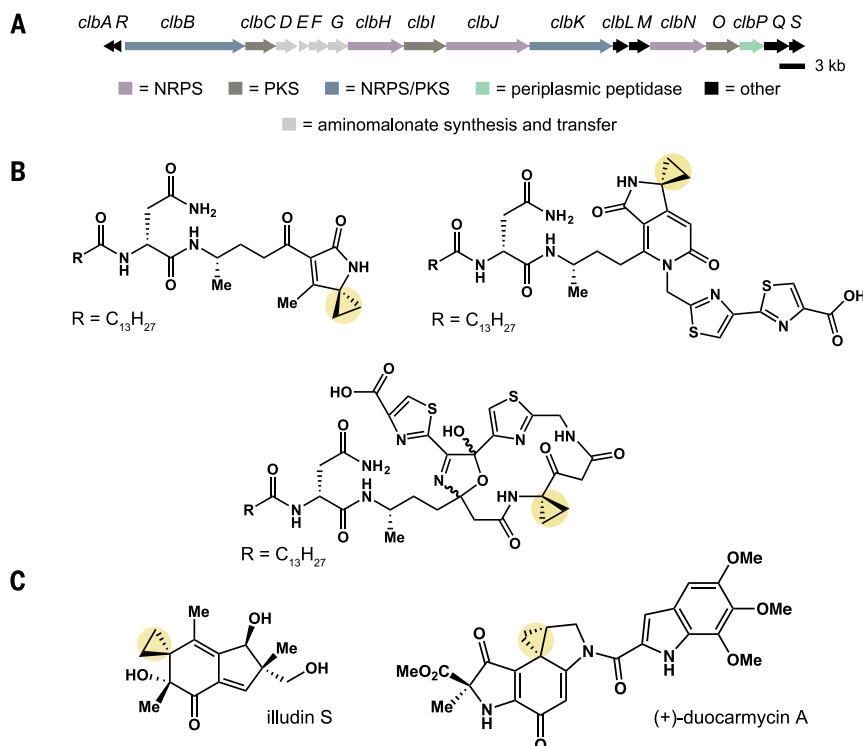


Fig. 1. *pks*⁺ *E. coli* synthesize cyclopropane-containing metabolites that may alkylate DNA. (A) The *pks* genomic island. Open reading frames encoding nonribosomal peptide synthetase (NRPS, purple), polyketide synthase (PKS, brown), hybrid NRPS-PKS (blue), peptidase (ClbP, green), aminomalonate synthesis and transfer (gray), and other (black) enzymes are highlighted. (B) Selected cyclopropane-containing candidate precolibactins isolated and structurally characterized from *pks*⁺ Δ *clbP* *E. coli*, which lacks *clbP*, including lactam, pyridone, and macrocyclic scaffolds. Me, methyl. (C) Illudin S and (+)-duocarmycin A are DNA alkylating metabolites that contain a cyclopropane ring.

an observed DNA adduct can allow for the determination of its elemental composition, and the triggered MS² and MS³ fragmentation spectra provide additional structural information about the modified base. We first used this DNA adductomic approach to detect characterized DNA adducts induced by illudin S, a cytotoxic agent that alkylates DNA upon cellular metabolic activation (28). LC-MS³ DNA adductomic analysis of hydrolyzed DNA obtained from HeLa cells exposed to either illudin S or dimethyl sulfoxide (DMSO) identified a known illudin-derived adduct (28) with a mass/charge number ratio (*m/z*) of 384.2030 [M+H]⁺ only in the illudin-treated cells, confirming the utility of this approach for adduct detection in our model (fig. S1).

Discovery of DNA adducts in mammalian cells and mice exposed to *pks*⁺ *E. coli*

We next investigated whether this method could identify putative colibactin-DNA adducts. First, we isolated DNA from HeLa cells transiently infected with either *pks*[−] or *pks*⁺ *E. coli*. After DNA hydrolysis, we performed a comparative, untargeted LC-MS³ DNA adductomic screen of these samples. This analysis revealed two putative DNA adducts (**1** and **2**) that accumulated only in the cells treated with *pks*⁺ *E. coli* (Fig. 2B).

We also detected these adducts in a colonic epithelial cell line exposed to *pks*⁺ *E. coli* and in HeLa cells exposed to native colibactin-producing strains (figs. S2 and S3). Adducts **1** and **2** eluted at 16.92 and 17.50 min, respectively, and exhibited a *m/z* of 540.1765 [M+H]⁺ (Fig. 2C). Both peaks triggered MS³ fragmentation events upon observation of the neutral loss of adenine (135.0542 u) in the MS² fragmentation spectra (Fig. 2C), indicating that these compounds were adenine adducts. To confirm adducts **1** and **2** were *pks*-associated, we repeated the cell infection assays described above but included individual stable isotope-labeled amino acids known to be used by the *pks* NRPS-PKS assembly line and integrated into *pks*-associated metabolites (10, 12, 13). These experiments revealed that the expected building blocks L-[2,3-¹³C₂]Ala, L-[1-¹³C]Met, [1,2-¹³C₂]Gly, and L-[1-¹³C]Cys were incorporated into the two adducts (figs. S4 to S8). While this work was in revision, Herzon and co-workers identified a putative DNA adduct with the same mass in plasmid DNA exposed to *pks*⁺ *E. coli* in vitro, thus further confirming our findings (29).

Next, we sought to determine whether adducts **1** and **2** could be detected in mice exposed to colibactin-producing *E. coli*. Germ-free wild-type C57BL/6J mice were inoculated with either *pks*[−] or *pks*⁺ *E. coli*. After 2 weeks, colonic epithelial

cells were harvested, DNA was isolated from the cells, and adduct formation was assessed using LC-tandem mass spectrometry (LC-MS/MS) (Fig. 2D). Both strains colonized the mice to a similar extent as assessed by fecal colony counts (Fig. 2E and table S1). We detected adducts **1** and **2** only in the mice colonized with *pks*⁺ *E. coli* (Fig. 2, F and G, fig. S9, and table S2). These results show that the colibactin-mediated DNA damage observed in human cell lines also occurs within a genetically intact host in the absence of exogenous carcinogens or inflammatory mediators. Furthermore, these data suggest that these adducts are biomarkers for *pks*⁺ *E. coli* exposure. Overall, this experiment provides the first direct support for DNA alkylation playing a critical role in colibactin's genotoxicity in vivo.

Structural characterization of the colibactin-derived DNA adducts

Further analysis of the LC-MS³ data revealed preliminary information about the structure(s) of adducts **1** and **2**. The high-resolution accurate-mass measurement of *m/z* 540.1765 [M+H]⁺ yielded a molecular formula of C₂₃H₂₅N₉O₅S (calculated, 540.1772) with 16 degrees of unsaturation. MS² fragmentation of **1** and **2** in high-resolution mode displayed major fragment ions of *m/z* 522.1665 [M+H-H₂O]⁺, 387.1118 [M+H-Ade-H₂O]⁺, 344.1060, and 229.0970. The shared fragmentation spectra indicated that these compounds were likely stereoisomeric (fig. S10). Using this information and our MS² fragmentation data, we proposed potential structures for the in vivo-derived colibactin-DNA adducts that were analogous to a recently characterized, chemically unstable “model colibactin”-thiol adduct but that contained an extra hydroxyl group (fig. S11) (25). However, these adducts' low abundance in cells precluded further isolation and structural characterization efforts.

To elucidate the structures of adducts **1** and **2**, we accessed authentic standards by chemically synthesizing new colibactin mimics and reacting them with calf-thymus DNA (ctDNA) (Fig. 3A). We prepared carboxylic acid-containing cyclopropane **3** in seven steps using a route developed to access other colibactin mimics (figs. S12 to S28 and 29A) (22). On the basis of the proposed structures of the adducts identified in *pks*⁺ *E. coli*-treated HeLa cells, **3** could react with ctDNA to give **1** and **2** directly. However, cyclopropane **3** generated only trace amounts of detectable adducts when incubated with ctDNA (fig. S30) and minimally sheared DNA at 1 mM concentration (fig. S31A). Hypothesizing that an unfavorable electrostatic interaction between the carboxylate of **3** and the negatively charged phosphate backbone of DNA greatly reduced its reactivity, we masked the carboxylic acid as an ethyl ester (figs. S12 to S19, S29B, and S32 to S35). Cyclopropane **4** was ~100-fold more potent than **3** in a DNA shearing assay and induced both G₂/M cell cycle arrest and DNA DSBs in treated HeLa cells (fig. S31B and S36 to S38). LC-MS analysis of a ctDNA reaction with **4** revealed three major products of

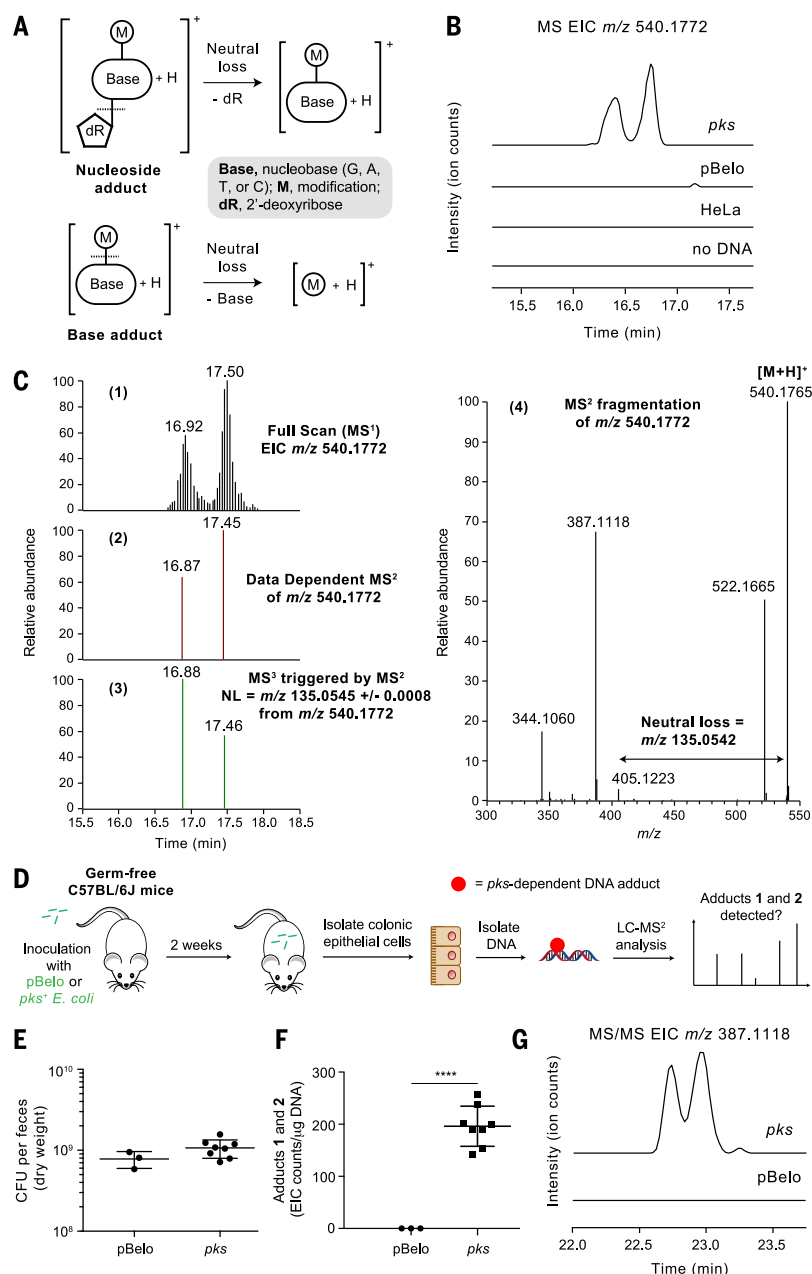


Fig. 2. High-resolution accurate-mass LC-MS³ DNA adductomic analysis identifies DNA adducts in HeLa cells and mice exposed to pks⁺ *E. coli*. (A) Structural features of DNA adducts and detection by neutral-loss monitoring. (B) Full scan extracted ion chromatogram (EIC) of DNA adducts **1** and **2** (m/z 540.1772) in HeLa cells exposed to colibactin-producing *E. coli* and negative controls (HeLa cells exposed to non-colibactin-producing pBeloBAC *E. coli*, HeLa cells alone, or when no DNA was present). (C) DNA adductomic analysis of adducts **1** and **2**. (1) Full scan EIC of DNA adducts **1** and **2** (m/z 540.1772). (2) Signal corresponding to the data dependent MS² events [retention time (RT) = 16.87 and 17.45 min]. (3) Signal corresponding to MS³ events (RT = 16.88 and 17.46 min) triggered by the neutral loss of adenine. (4) MS² mass spectrum resulting from fragmentation of m/z 540.1772, which triggered the MS³ event. (D) Flowchart of the experiment detecting DNA adducts **1** and **2** in mouse colonic epithelial cells. (E) Bacterial load in the feces of mice colonized with pBelo ($n = 3$) or pks⁺ *E. coli* ($n = 8$) for 2 weeks. CFU, colony forming units. (F) EIC counts of DNA adducts **1** and **2** per µg of DNA in colonic epithelial cells isolated from mice colonized with pBelo ($n = 3$) or pks⁺ *E. coli* ($n = 8$) for 2 weeks. EIC counts were determined by area-under-the-curve integrations of the most abundant MS² fragmentation ion (m/z 387.1118 ± 0.0008) of the adducts **1** and **2** precursor ion (m/z 540.1772). (G) Representative MS/MS EIC of DNA adducts **1** and **2** (m/z 387.1118 [M+H-Ad-H₂O]⁺), the most abundant fragment ion of m/z 540.1772. Each symbol in (E) and (G) represents an individual mouse; error bars represent mean ± SEM; **** $P < 0.0001$ (unpaired Student's *t* test).

m/z 552.2125 [M+H]⁺ (**5**) and m/z 568.2076 [M+H]⁺ (**6** and **7**) (figs. S30 and S39). MS² fragmentation confirmed that these compounds were adenine adducts that only differed by the presence of one oxygen atom (figs. S40 and S41). The “nonoxidized” adduct **5** (m/z 552.2135) was unstable at room temperature and slowly converted to a pair of “oxidized” adducts (**6** and **7**) (m/z 568.2081) over the course of 2 days (figs. S42 and S43). Further analysis of the fragmentation data indicated that **6** and **7** contained a hydroxyl group and a fragment ion (m/z 229.0970) identical to that of the in vivo-derived adducts **1** and **2** (fig. S44). Because **6** and **7** were stable and possessed an MS² fragmentation pattern matching those of the adducts detected in *pks*⁺ *E. coli*-treated HeLa cells, we targeted these compounds for isolation and structural characterization.

We isolated and purified **6** and **7** from multiple small-scale ctDNA alkylation reactions to give approximately 1 mg of pure material. Analysis by one-dimensional (¹H) and two-dimensional (gCOSY, gHSQC, gHMBC, and ROESY) nuclear magnetic resonance (NMR) (figs. S45 to S52 and tables S3 and S4), as well as DP4 computational analysis (30) (fig. S53 and tables S5 to S7), revealed a 1:1 diastereomeric mixture of a single adduct that contains a 5-hydroxypyrrrolidin-2-one ring system with an attached N3-substituted adenine ring (Fig. 3B). The N3-adenine and hemiaminal substitution assignments were supported by key ¹H-¹³C heteronuclear multiple bond (HMBC) and through-space ¹H-¹H correlations. Interestingly, the observed preference for N3-adenine alkylation resembles that of other cyclopropane-containing DNA alkylating agents (28, 31).

We hypothesized that the structures of the monoadducts obtained in vitro (**6** and **7**) and the adducts identified from *pks*⁺ *E. coli*-treated cells (**1** and **2**) differed only in the presence of an ester versus carboxylic acid functional group on the basis of their shared MS² fragmentation patterns. To confirm the structure of the adducts generated in vivo (**1** and **2**), ester-containing adducts **6** and **7** were hydrolyzed using pig liver esterase to give an authentic standard of the corresponding carboxylic acids (fig. S54). LC-MS¹ and LC-MS² analysis revealed that this standard possessed the same m/z , retention time, and MS² fragmentation pattern as the adducts (**1** and **2**) identified in *pks*⁺ *E. coli*-treated mammalian cells (Fig. 3C and fig. S55), thereby confirming their chemical structures. On the basis of the observed reactivity of the nonoxidized adduct **5** in vitro and the reactivity of related synthetic compounds (22, 25), we propose that the 5-hydroxypyrrrolidin-2-one ring found in adducts **1** and **2** likely arises from oxidation of an initial, chemically unstable enamide-containing adduct (fig. S56).

Successful characterization of monoadducts **1** and **2** confirms that exposure of host cells to *pks*⁺ *E. coli* results in DNA alkylation. This finding provides direct information about colibactin's structure, resolves questions surrounding the active genotoxin's electrophilic cyclopropane and mode of activation, and allows us to propose a mechanism for how exposure to colibactin

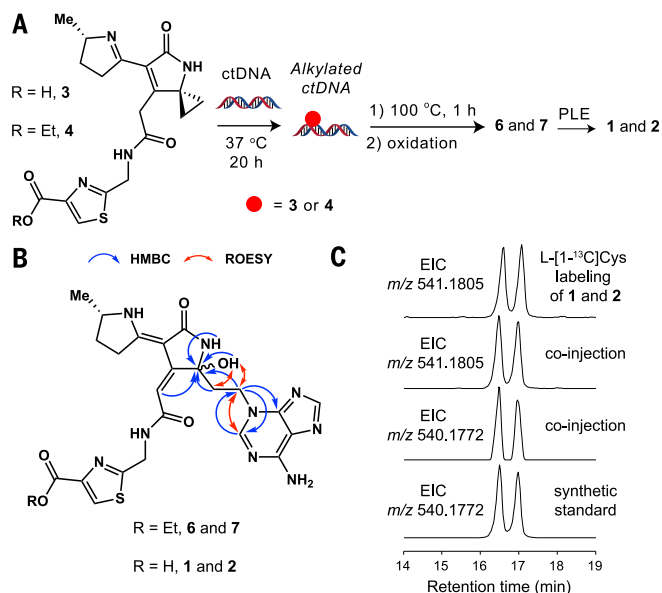


Fig. 3. Comparing DNA adducts generated in vivo with a synthetic standard confirms their chemical structures. (A) In vitro DNA alkylation reaction used to generate a synthetic standard of adducts **1** and **2**. PLE, pig liver esterase. (B) Chemical structures of diastereomeric DNA adducts **6** and **7** showing key two-dimensional NMR correlations that support the hemiaminal and N3-adenine assignments. (C) EICs of the L-[1-¹³C]Cys-labeled in vivo adducts **1** and **2** (m/z 541.1805), co-injection of synthetic standard (m/z 540.1772) with in vivo adducts **1** and **2** (m/z 541.1805 and residual unlabeled m/z 540.1772), and synthetic standard (m/z 540.1772).

leads to DNA damage. The precolibactins, isolated to date, possess a variety of cyclopropane-containing scaffolds, including linear structures (**13**), an unsaturated lactam (**10–12**), a pyridone (**13, 14**), and a macrocycle (**15**). The structures of **1** and **2** strongly suggest that the cyclopropane in colibactin is conjugated to an α,β -unsaturated imine and is not embedded within a linear framework or pyridone. Furthermore, our findings provide in vivo evidence that cleavage of pre-colibactin by peptidase ClbP generates the active colibactin genotoxin by triggering an intramolecular cyclodehydration to form an α,β -unsaturated imine, enhancing the reactivity of the cyclopropane toward DNA (Fig. 4A) (**10, 11, 22**).

Exposure to *pks*⁺ *E. coli* generates interstrand cross-links in cells

Multiple lines of evidence suggest that adducts **1** and **2** are unlikely to represent the immediate product of DNA alkylation with the mature colibactin genotoxin and instead arise from degradation of a larger monoadduct and/or cross-linked adduct. First, the carboxylic acid-containing model colibactin **3** is a poor DNA alkylating agent. Second, although **3** could be generated using a subset of the colibactin biosynthetic enzymes, the entire NRPS-PKS assembly line is essential for genotoxicity (**2**). Finally, recent work

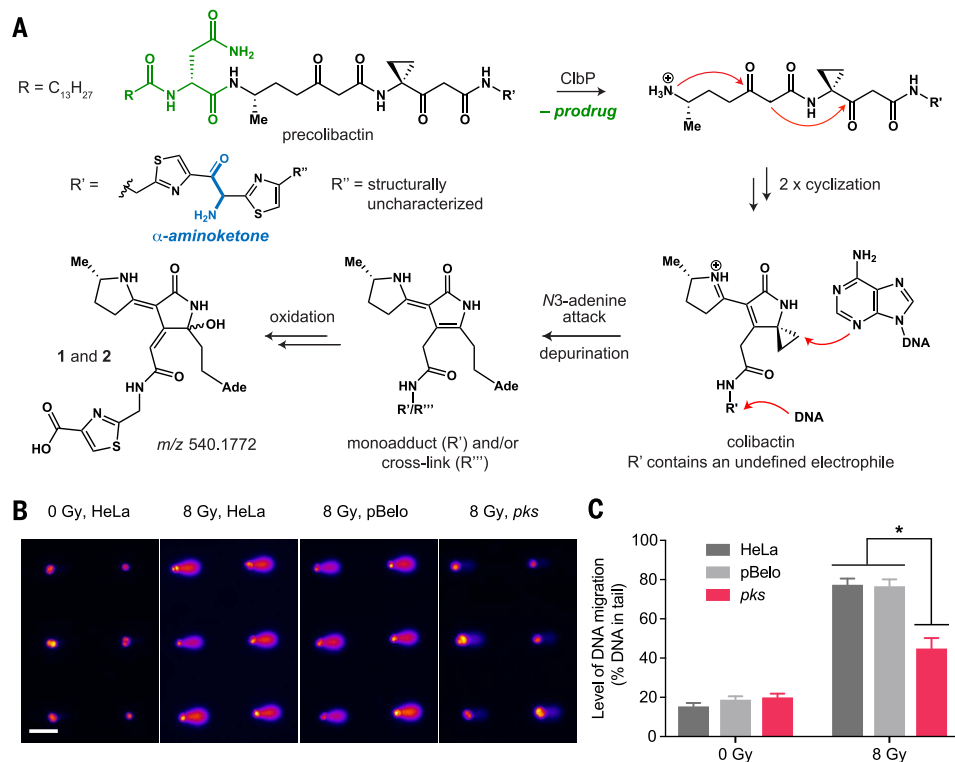


Fig. 4. The characterized DNA adducts may derive from a colibactin-DNA interstrand cross-link. (A) Proposed model for colibactin DNA alkylation and formation of DNA adducts **1** and **2**. Ade, adenine. (B) Arrayed microwell comets from untreated HeLa cells and HeLa cells treated with pBelo or *pks*⁺ *E. coli* for 1 hour at 37 °C [multiplicity of infection (MOI) = 1000]. Scale bar indicates 100 μ m.

(C) Quantification of the percentage of DNA in tail from untreated HeLa cells and HeLa cells treated with pBelo or *pks*⁺ *E. coli* for 1 hour at 37 °C (MOI = 1000). Data points and error bars represent mean $\pm$ SEM, respectively, of three independent experiments. Student's *t* test was performed to compare each treated dose to the corresponding negative controls (**P* < 0.05).

reported the accumulation of a putative interstrand cross-link in DNA incubated with *pks*⁺ *E. coli* as assessed by gel electrophoresis and found that cell lines deficient in interstrand cross-link repair were more sensitive to colibactin-producing *E. coli* (32).

To explore the correlation between DNA cross-linking and the formation of **1** and **2**, we used a modified alkaline single-cell gel electrophoresis assay (comet assay) (33) to assess the presence of interstrand cross-links in cells exposed to *pks*⁺ *E. coli*. This assay utilizes the high degree of strand breaks induced by γ radiation to measure interstrand cross-link formation. Unlike monoadducts, interstrand cross-links inhibit the denaturation of DNA under alkaline conditions and therefore decrease the level of DNA migration, reducing the ability to detect radiation-induced strand breaks. We first tested whether CometChip, a high-throughput platform and more robust version of the comet assay (34, 35), could detect interstrand cross-links generated by cisplatin, a bifunctional cross-linking agent. Cells were exposed to varying concentrations (0 to 200 μ g/ml) of cisplatin and then analyzed for cross-links 6 hours after drug treatment. As expected, cisplatin caused a significant decrease in DNA migration in treated cells, thus confirming the utility of this assay (fig. S57).

Next, we applied CometChip to investigate whether interstrand cross-links are formed in HeLa cells exposed to colibactin at the same time point at which we detected adducts **1** and **2**. HeLa cells were infected with *pks*⁺ *E. coli* for 1 hour, and cross-link formation was measured immediately afterward. In *pks*⁺ *E. coli*-treated HeLa cells exposed to γ irradiation [8 gray (Gy)], we detected a significant level of cross-links as indicated by the 32% decrease in DNA tail moment compared to both controls (Fig. 4, B and C). By contrast, we observed minimal strand breaks in non- γ -irradiated *pks*⁺ *E. coli*-treated cells. Thus, these results indicate that interstrand cross-links are present in the *pks*⁺ *E. coli*-treated HeLa cells from which we isolated **1** and **2**. However, we could not identify any masses corresponding to putative interstrand cross-links in our untargeted DNA adductomics datasets, suggesting that they may be unstable to our isolation, purification, or MS conditions.

Possible origins of colibactin-derived DNA adducts

Knowledge of colibactin biosynthesis suggests a mechanism for how DNA adducts or cross-links degrade to form **1** and **2**. Bioinformatic analyses of the NRPS-PKS assembly line and isolation of precolibactins have indicated that colibactin likely contains a bithiazole (13, 14) and/or a related ring system with an α -aminoketone inserted between two thiazole rings (Fig. 1B) (15). However, searching our untargeted DNA adductomics datasets did not reveal masses corresponding to putative bithiazole or α -aminoketone-containing colibactin-DNA adducts. Because adducts **1** and **2** contain only one thiazole heterocycle, we propose that they derive from oxidative C–C cleavage

of a larger, α -aminoketone-containing colibactin-DNA monoadduct or cross-link (Fig. 4A). Whereas bithiazole rings are stable, α -aminoketones undergo oxidative C–C bond cleavage in the presence of reactive oxygen species to give carboxylic acids (36). Therefore, the structures of **1** and **2** strongly suggest that the active genotoxin contains an α -aminoketone, a positively charged functional group that may enhance colibactin's affinity for DNA and thus increase its potency (22). Further experiments will be needed to clarify the nature of the interstrand cross-link, the origin and timing of the proposed oxidative degradation event, and how the specific lesions(s) generated by colibactin lead to the formation of DBSs.

Conclusions

We have presented direct evidence that the gut bacterial genotoxin colibactin alkylates DNA in vivo. The ability of *pks*⁺ *E. coli* to generate DNA adducts in mammalian cells and in mice strengthens support for the involvement of colibactin in cancer development or progression because misrepaired monoadducts and cross-linked adducts may generate mutations in oncogenes or tumor suppressor genes, contributing to tumorigenesis (37, 38). Our findings will enable efforts to decipher the molecular details of this process. Importantly, this work has also uncovered a candidate metabolite biomarker of colibactin exposure and cancer risk. The ability to directly assess whether exposure to colibactin has occurred in animal models and human patients will help address the critical question of whether *pks*⁺ *E. coli* contribute to colorectal carcinogenesis in patient cohorts (39). Finally, this study showcases the use of untargeted DNA adductomics for the identification and elucidation of unknown gut microbial-derived DNA modifications, highlighting the power of emerging analytical techniques in studying human microbiota metabolites and host-microbiota interactions.

Materials and methods summary

Our methods for the preparation of *E. coli* strains for cell infection and isotope labeling, bacterial monoclonization of germ-free mice, identification of colibactin-derived DNA adducts by DNA adductomics, synthetic preparation of model colibactins and synthetic standards of the colibactin-derived DNA adduct, structural characterization of compounds by one- and two-dimensional NMR methods and DP4 computational analysis, and assessment of interstrand cross-link formation using the CometChip assay are provided in the supplementary materials. Additional information about our protocols, including references to the supplementary materials, can be found throughout the main text.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/eaar7785/suppl/DC1
Materials and Methods
Figs. S1 to S57
Tables S1 to S7
References (40, 41)

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RESEARCH ARTICLE

STRUCTURAL BIOLOGY

A human postcatalytic spliceosome structure reveals essential roles of metazoan factors for exon ligation

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During exon ligation, the *Saccharomyces cerevisiae* spliceosome recognizes the 3'-splice site (3'SS) of precursor messenger RNA (pre-mRNA) through non-Watson-Crick pairing with the 5'SS and the branch adenosine, in a conformation stabilized by Prp18 and Prp8. Here we present the 3.3-angstrom cryo-electron microscopy structure of a human postcatalytic spliceosome just after exon ligation. The 3'SS docks at the active site through conserved RNA interactions in the absence of Prp18. Unexpectedly, the metazoan-specific FAM32A directly bridges the 5'-exon and intron 3'SS of pre-mRNA and promotes exon ligation, as shown by functional assays. CACTIN, SDE2, and NKAP—factors implicated in alternative splicing—further stabilize the catalytic conformation of the spliceosome during exon ligation. Together these four proteins act as exon ligation factors. Our study reveals how the human spliceosome has co-opted additional proteins to modulate a conserved RNA-based mechanism for 3'SS selection and to potentially fine-tune alternative splicing at the exon ligation stage.

The spliceosome excises introns from precursor messenger RNAs (pre-mRNAs) to produce mature mRNA in two sequential transesterifications—branching and exon ligation—catalyzed at a single active site (1–3). The spliceosome assembles de novo on each pre-mRNA from component small nuclear ribonucleoproteins (snRNPs) and undergoes numerous conformational changes mediated by trans-acting proteins and DEAx/H-box adenosine triphosphatases (ATPases) (4). A series of cryo-electron microscopy (cryo-EM) structures of *Saccharomyces cerevisiae* (hereafter referred to as yeast) spliceosomes at different stages of assembly, catalysis, and disassembly have rationalized decades of biochemical and genetic data and have provided considerable mechanistic insights into how the spliceosome achieves these two trans-esterification reactions (1, 5–9). During initial assembly, the U1 snRNP base-pairs with the 5'-splice site (5'SS), whereas the U2 snRNP forms the branch helix through pairing around the branch point (BP) adenosine. Prespliceosome formation, involving minimal interaction between the U1 and U2 snRNPs in yeast, brings the 5'SS and the BP sequence into one assembly. In mammals, formation of the prespliceosome is promoted and regulated by many alternative splicing factors (10, 11). The prespliceosome then associates with the U4/U6-U5 tri-snRNP to form the pre-B com-

plex, which is converted via B to B^{act} when U1 and U4 snRNPs dissociate by the activities of Prp28 and Brr2, which is followed by binding of the multisubunit Prp19-associated (NTC) and Prp19-related (NTR) complexes. The 5'SS is handed off to the U6 small nuclear RNA (snRNA), and the catalytic core is formed during this conversion. The catalytic core of the spliceosome comprises U6 and U2 snRNAs folded into a compact structure that binds two catalytic divalent ions (12–14). The 5'SS is positioned precisely at the catalytic metal ions by pairing between the conserved 5'-intron sequence, GUAUGU, and the ACAGAGA sequence of U6 snRNA and between the 5'-exon and U5 snRNA loop I (15, 16). During Prp2-induced remodeling to B*, the branch helix is docked into the active site by the branching factors Cwc25 and Yju2, which allows the 2'-hydroxyl group of the BP adenosine to attack the 5'SS, producing the free 5'-exon and a lariat intron-3'exon intermediate (1). Prp16-induced dissociation of the branching factors from the resulting C complex promotes rotation of the branch helix out of the active site (17). Exon ligation factors lock the branch helix into its new position in the resulting C* complex (5, 6). The 3'SS is positioned at the catalytic metal ions by non-Watson-Crick base-pairing between the last intron nucleotide G and the first intron nucleotide G, as well as between the penultimate intron nucleotide A and the BP adenosine. This configuration allows the 3'-hydroxyl group of the 5'-exon to attack the 3'SS, ligating the 5'- and 3'-exons into mRNA (7–9). The DEAH-box ATPase Prp22 then releases the resulting mRNA from the postcatalytic P complex

(18, 19), and finally the ATPase Prp43 disassembles the spliceosome for new rounds of splicing (1–3).

Human spliceosomes are larger than their yeast counterparts and contain many additional proteins (3, 20, 21). Cryo-EM structures of the human spliceosomes captured at near-atomic resolution in different states confirm that the general architecture of the spliceosome is largely conserved between yeast and humans and reveal how some additional human proteins are integrated into the conserved architecture of the spliceosome (22–27). However, the functions of these proteins have not been determined experimentally. It is also not known if these proteins are constitutive components of the human spliceosome or whether some of them regulate alternative splicing of subsets of pre-mRNAs in a tissue-specific manner. Here we report the cryo-EM structure of the human postcatalytic spliceosome, which shows that the 3'SS is recognized through RNA-RNA interactions conserved between humans and yeast. Our high-resolution structure reveals that four proteins, not previously observed in human spliceosome structures, stabilize the branch helix and the docked 3'SS to facilitate exon ligation.

Purification and overall structure of the human P complex

The P-complex spliceosome was assembled on MINX pre-mRNA in HeLa nuclear extract supplemented with recombinant hPrp22 (DHX8) mutant (K594A; see supplementary note 1 and fig. S1) to prevent release of ligated exons. Oligonucleotide-directed RNase H digestion was targeted to the region of the 3'-exon protected only when the 3'SS is docked into the active site. The resulting P complex was affinity-purified on amylose-resin by using three MS2 aptamers attached to the 3'-exon to eliminate contaminating C* complex (supplementary methods; figs. S1 and S2) (7).

The overall architecture of the human P complex obtained by cryo-EM reconstruction at 3.3 Å resolution (supplementary materials PyMOL session, figs. S2 to S5) is similar to that of the human C* complex determined at an average resolution of 3.76 Å (22) and 5.9 Å (26) (Fig. 1). The higher resolution of our cryo-EM density map of the human P complex allowed us to build more-complete models of proteins in the peripheral region (table S2) and parts of four additional proteins (Cactin, FAM32A, SDE2, and NKAP) not present in *S. cerevisiae* (Fig. 1, B and C, and figs. S5 and S6). The remaining parts of these proteins are predicted to be largely disordered. The densities for Cactin and FAM32A were partially visible in the map of the C* complex (22) but were not of sufficient quality for model building. The higher-resolution map of our P complex allowed us to build the C-terminal half of FAM32A based on density alone, but the highly charged N-terminal half is disordered (fig. S6).

A conserved 3'SS recognition mechanism

The RNA-based active site of the human P complex is essentially unchanged compared to C*,

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with the U2 and U6 snRNAs forming a triple helix that binds two catalytic Mg^{2+} ions (fig. S7). In the human P complex, the newly formed mRNA remains bound at the active site through its 5'-exon pairing to U5 snRNA (fig. S7A). The new phosphodiester bond connecting the 5'-exon to the first two nucleotides of the 3'-exon is clearly visible, confirming that our sample represents the genuine P complex (fig. S5B). Clear density

extending from the intron G(+1) and the BP adenosine could be modeled as the last three nucleotides of the 3'SS (Fig. 1E and fig. S5B). As in yeast, the Hoogsteen edge of the 3'SS G(-1) forms a base pair with the Watson-Crick edge of the 5'SS G(+1). Additionally, N7 of the 3'SS A(-2) forms an H-bond with N6 of the BP adenosine. Thus, the 3'SS is recognized, as in yeast (Fig. 1, E and F), through pairing with the 5'SS and the

BP adenosine. The 5'SS U(+2) pairs with the U6 snRNA A51, which stacks on the 3'SS G(-1), an interaction that was not modeled in the human C* complex (22) and which allows the 3'-hydroxyl of 3'SS G(-1) to project into the active site. Docking

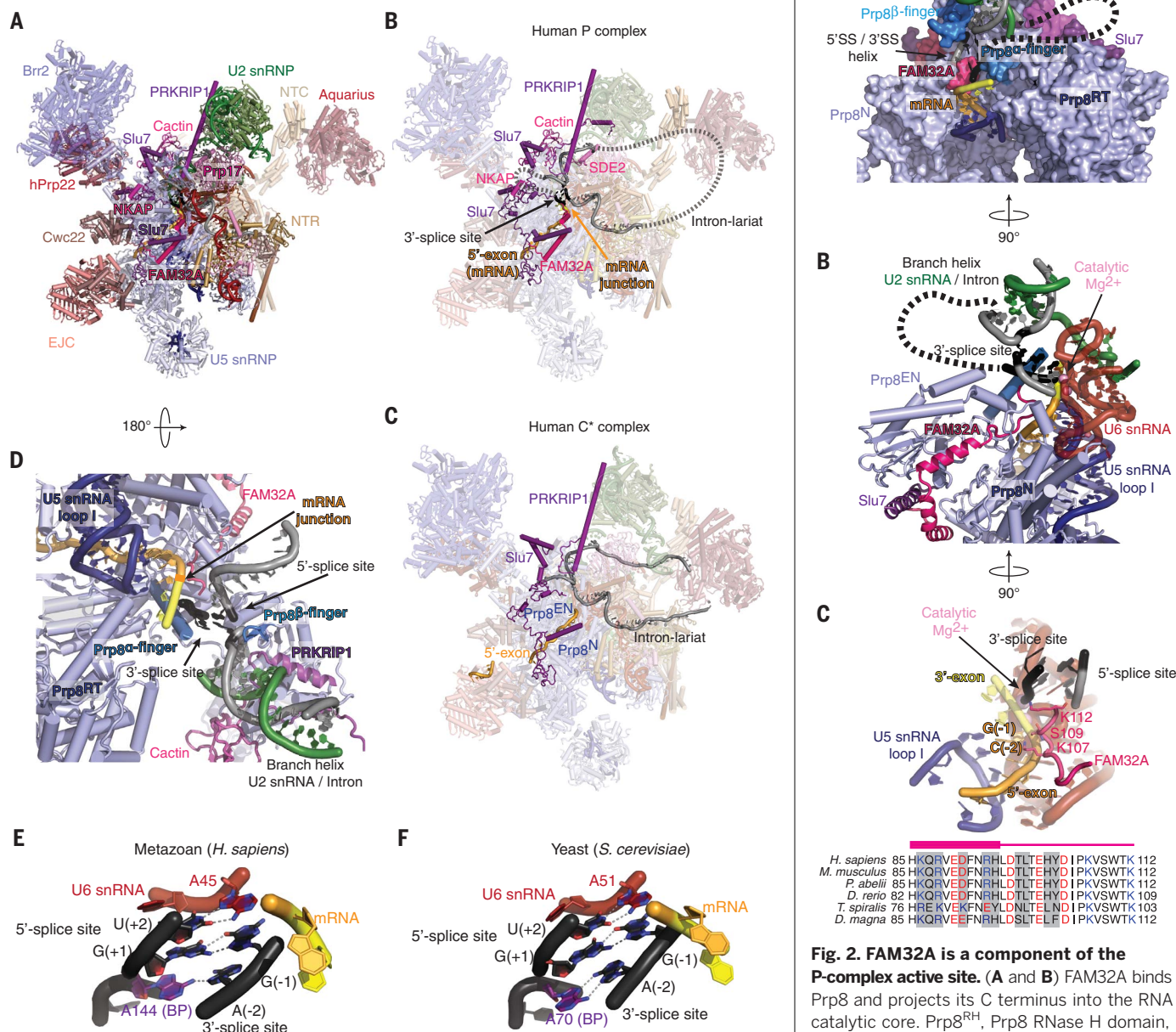


Fig. 1. Structure of a human P complex reveals unexpected exon ligation factors.

(A) Overview of the human P complex spliceosome complex. EJC, exon junction complex; NTC, Prp19-associated complex; NTR, Prp19-related complex. (B and C) Comparison of the P (present work) and C* (22) complexes reveals previously unknown factors. The presence of mRNA and the docked 3'-splice site in our P-complex structure are apparent. Dashed lines indicate possible path of the intron not visible in the density. The intron is shown in gray, the 5'-exon in orange, and the 3'-exon in yellow. Prp8^{EN}, Prp8 endonuclease domain; Prp8^N, Prp8 N-terminal domain. (D) Binding of the substrate in the active site cavity of P complex. Prp8^{RT}, Prp8 reverse-transcriptase domain. (E) The 3'SS is recognized by the 5'SS and the BP adenosine in the human P complex. (F) 3'SS recognition in the yeast P complex (7).

Fig. 2. FAM32A is a component of the P-complex active site. (A and B) FAM32A binds Prp8 and projects its C terminus into the RNA catalytic core. Prp8^{RT}, Prp8 RNase H domain, Prp8^N, N-terminal domain of Prp8. (C) FAM32A stabilizes the 5'-exon onto U5 snRNA loop I, in proximity to the docked 3'SS. The highly conserved FAM32A C terminus across metazoans is apparent; variable residues are shaded gray. Dashed lines indicate possible path of the intron not visible in the density. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

of the 3'SS onto the 5'SS is stabilized by the Prp8 alpha-finger and beta-finger—another feature similar to that of yeast (Fig. 1D). However, Prp18—which in yeast projects into the active site and stabilizes the intron upstream of the 3'SS at positions –3 to –5—was not observed in our map and was not detected by mass spectrometry either in our sample or in previous mass-spectrometric studies of C* and P complexes (20, 26, 27). Indeed, beyond the 3'SS C(–3), the intron becomes disordered in our map. The remaining nucleotides between the 3'SS and the branch helix loop out of the spliceosome, and their path is likely guided by mammalian-specific proteins, as described below.

FAM32A is a metazoan-specific exon ligation factor

The most notable finding in our structure is that FAM32A (figs. S5C and S6), a poorly characterized protein of 13 kDa, binds between the endonuclease (EN) and N-terminal (N) domains of Prp8 and projects its C terminus deep into the active site (Fig. 2, A and B). Here FAM32A stabilizes the pairing between the 5'SS, the 3'SS,

and the BP adenosine together with the alpha-finger and beta-finger of Prp8 (Fig. 2A). The C terminus of FAM32A binds along the 5'-exon through direct contacts between K107 and S109 and the phosphates of C(–2) and G(–1), respectively, and stabilizes its base-pairing to loop I of U5 snRNA (Fig. 2C). The positively charged side chain of its C-terminal K112 extends into the space where the 5'SS, 3'SS, and BP come together to promote docking of the 3'SS (Fig. 2C). FAM32A is also known as ovarian tumor associated gene-12 (OTAG-12) and is down-regulated in a mouse model of ovarian tumor development (28). The OTAG-12 gene is expressed as three splice isoforms—OTAG-12a, OTAG-12b, and OTAG-12c—in mice (figs. S5C and S6 and supplementary note 2), and expression of the full-length OTAG-12b in ovarian cancer and human embryonic kidney 293 (HEK293) cells suppressed cell growth whereas OTAG-12c with N-terminal deletion or OTAG-12a with altered C-terminal sequence had no such effect. FAM32A (OTAG-12b, fig. S6B) bound in the P complex promotes mRNA formation for proapoptotic genes, acting as a tumor suppressor. Indeed, the entire C terminus of FAM32A is es-

entially invariant in metazoans from zebrafish to humans (Fig. 2C), consistent with a role in regulating splicing.

Depletion of FAM32A from HeLa nuclear extracts impaired exon ligation (Fig. 3, A to E, and fig. S8, A to C), causing accumulation of cleaved 5'-exon at the C* stage (fig. S8, D and E). Recombinant FAM32A restored efficient mRNA formation (Fig. 3D and fig. S8, B and C), demonstrating that FAM32A promotes splicing by facilitating exon ligation, in agreement with our structure. Ultraviolet (UV) cross-linking using pre-mRNA containing a single 4-thioU substitution at position –2 of the 5'-exon (Fig. 3, C, D, F, and G) produced two major cross-links (Fig. 3, F and G). The one above 200 kDa represents Prp8, whereas the cross-link between 15 and 25 kDa was confirmed to be FAM32A by depletion and addition of slightly larger, tagged FAM32A (Fig. 3, G and H). P complexes assembled in FAM32A-depleted extracts contained mostly lariat-intermediate and cleaved 5'-exon, which cross-linked to residual FAM32A, demonstrating that FAM32A also binds the 5'-exon in the precatalytic C* complex (Fig. 3, F and G). Thus, FAM32A is a bona fide exon ligation factor that stabilizes docking of the 3'SS into the active site and promotes splicing in mammals.

NKAP and FAM32A stabilize Slu7 binding

As in yeast, Slu7 rigidifies the C*/P conformation by binding across the Prp8 EN and N domains (Fig. 1C and 4, A to C). Binding of the central region of Slu7 to the Prp8 EN domain is stabilized by FAM32A (Fig. 2B), whereas nuclear factor κB-activating protein (NKAP)—a previously unidentified factor—promotes binding of the Slu7 N terminus onto Prp8 (Fig. 4C and fig. S5D). NKAP is a 415-residue protein implicated in T cell development; it consists of highly charged repetitive sequences such as Ser-Arg and poly-Lys and is expected to be intrinsically disordered through almost its entire length. However, residues 329 to 358 form a short helix that bridges the N- and C-terminal fragments of Slu7 bound to Prp8 and stabilizes the P complex. Indeed, NKAP binds exon sequences genome-wide and associates with mRNA in vitro, and depletion of NKAP in vivo reduces splicing efficiency, consistent with a role in promoting mRNA formation (29).

Cactin, SDE2, and PRKRIP1 stabilize the branch helix

The branch helix is locked into position by the WD40 domain of Prp17, CDC5L (Ceft), and CRNKL1 (Clf1), as in yeast C* and P complexes (5, 7–9) (Fig. 4, D to G). Unexpectedly, the human P complex structure revealed that the branch helix is further secured in its exon ligation conformation by Cactin, SDE2, and PRKRIP1. Our cryo-EM map enabled us to build residues between 637 and 756 of Cactin, which folds into a β-sandwich domain. Its N-terminal region has long stretches of charged and polar amino acids, suggesting that these regions are intrinsically disordered. Its C terminus and a short α helix protruding from the

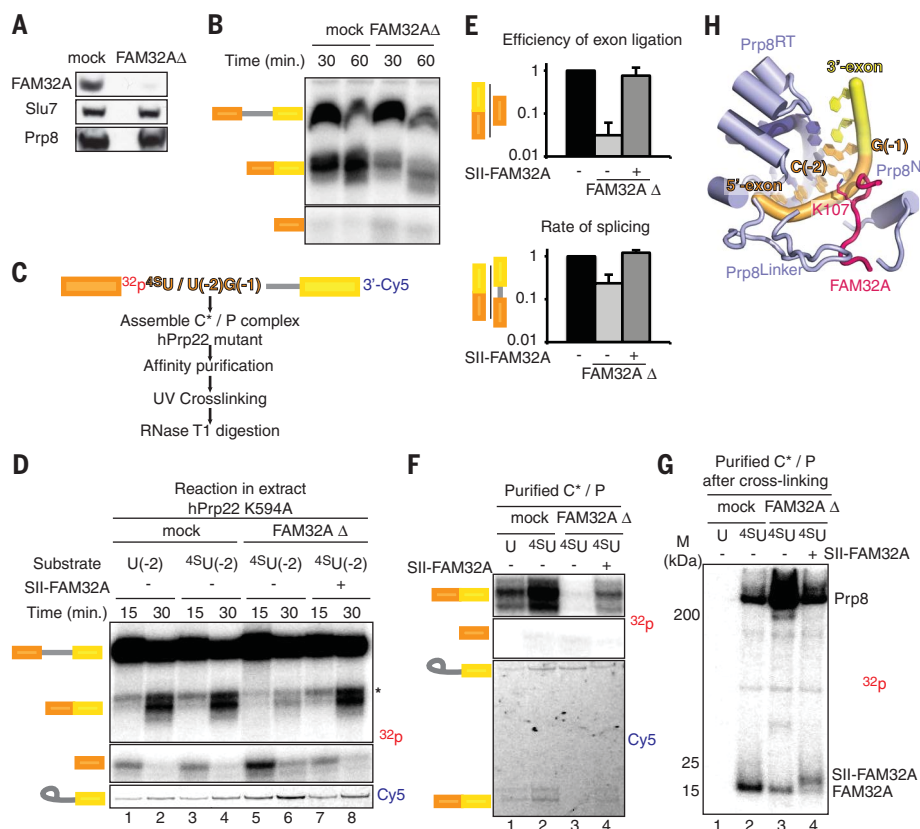


Fig. 3. FAM32A promotes exon ligation by binding the 5'-exon. (A and B) Depletion of FAM32A impairs exon ligation. (C) Overview of the UV cross-linking experiment. C(–2) was changed to U(–2) for these experiments. (D) FAM32A promotes exon ligation. (E) Effect of FAM32A depletion on exon ligation efficiency. Experiments were performed using a substrate with a single ³²P at U(–2) of the 5'-exon. Error bars represent SD (n = 3). (F) C* complexes accumulate in FAM32A-depleted extracts. Shown is RNA extracted from affinity-purified P complexes (see also fig. S8). (G) FAM32A cross-links to the 5'-exon. SDS-polyacrylamide gel electrophoresis of proteins labeled through cross-linking. SII-FAM32A, Strep-tactin-tagged FAM32A; ³²p, ³²P radioactive phosphate; ⁴⁵U, 4-thio-uridine. (H) Positioning of FAM32A and Prp8 around C(–2) of the 5'-exon rationalizes the cross-linking results.

β -sandwich domain interact with the Prp8 RNase H domain (Fig. 4, D and E), allowing Cactin to project a series of charged residues toward the branch helix, near the predicted path of the intron between the BP and the docked 3'SS (Fig. 4E), stabilizing 3'SS docking. Finally, a loop just before the C-terminal β strand of Cactin forms an extensive positively charged surface with the N-terminal region of CRNKL1 and with Cdc5L that surrounds the branch helix (Fig. 4, F and G). This surface is stabilized by an α helix of SDE2 that interacts with CRNKL1 and Cdc5L (Fig. 4G and fig. S9A). Our map also enabled us to build 30 additional residues of PRKRIP1 (22), which reveals its distinctive structure (fig. S9, B and C). The N-terminal residues 28 to 39 form an α helix that bridges between the U2 Sm ring and U2 snRNA at the tip of the branch helix. Together with the long C-terminal α helix bound to stem IV of U2 snRNA, it locks the branch helix into the exon ligation orientation (fig. S9B). The intervening loop inserts into the active site and interacts with the Prp8 RNase H domain and the C terminus of Cactin to stabilize the branch helix and promote exon ligation.

SDE2 is first synthesized as an inactive precursor containing an N-terminal ubiquitin-fold domain, which is cleaved to produce activated Sde2-C. Our structure shows that the N-terminal ubiquitin domain of unprocessed SDE2 would clash with the branch helix (Fig. 4, F and G), explaining why the full-length protein cannot be incorporated into the spliceosome, as shown in *Schizosaccharomyces pombe*. *S. pombe* cells that cannot produce Sde2-C show defects in splicing of the same specific introns as cells lacking Cactin (30), suggesting that binding of Cactin and Sde2 to the spliceosome is highly cooperative. Indeed, cells lacking Sde2-C show reduced Cactin binding to the spliceosome (30). The P-complex structure shows how SDE2 guides CRNKL1 to bind Cactin (Fig. 4G), thus rationalizing the functional observations in *S. pombe*.

Discussion

The human P-complex structure shows that the 3'SS is recognized and docked into the active site of the spliceosome on the basis of the same base-pairing interactions seen in the yeast P complex (7–9). Similarly to yeast, a set of conserved factors including the RNase H domain of Prp8, Prp17, and Slu7 rigidify the position of the branch helix in the P complex and promote 3'SS docking (7). Unexpectedly, our high-resolution map of human P complex enabled us to build four additional proteins—FAM32A, Cactin, SDE2, and NKAP, which have been identified by mass spectrometry but have not been found in any of the cryo-EM structures of human spliceosomes (22–26). Three of these factors—NKAP, Cactin, and Sde2—cooperate with PRKRIP1 to lock the branch helix in place in P complex (Fig. 5). Indeed, Cactin and Sde2 promote splicing of the same specific subset of introns in *S. pombe* (31), highlighting their role in exon ligation, although the basis of their specificity is not understood. Together these factors partially compensate for the absence of Yju2, which

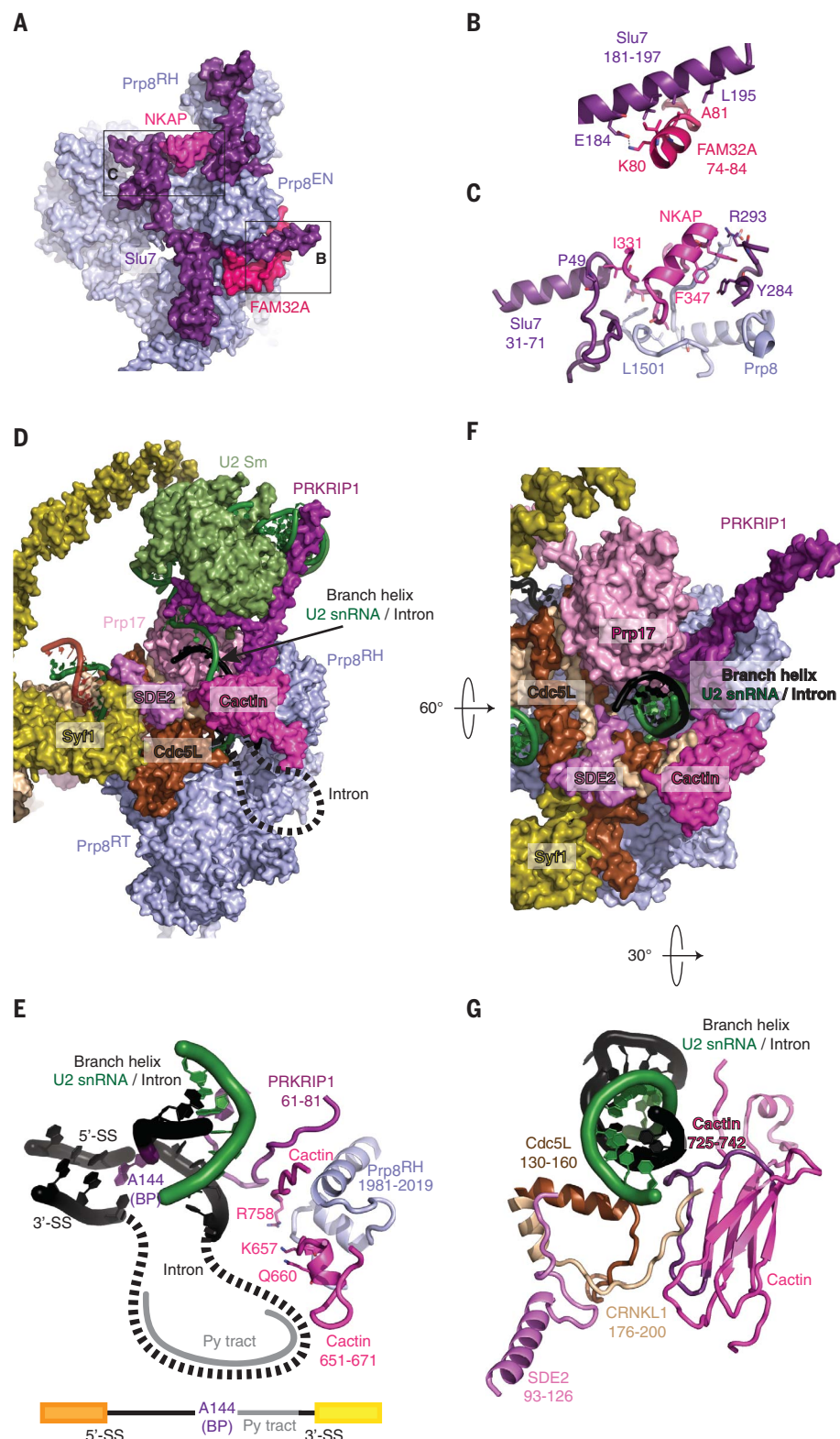


Fig. 4. Unexpected factors stabilize the P-complex conformation. (A to C) FAM32A and NKAP promote binding of Slu7 to the P complex. Prp8^{EN}, Prp8 endonuclease domain. (D and E) Cactin stabilizes the position of the branch helix for exon ligation. Dashed lines indicate possible path of the intron not visible in the density. Py tract, polypyrimidine tract. (F) Previously unidentified factors position the branch helix. (G) SDE2 promotes Cactin binding near the branch helix. The loop of Cactin that projects a positive surface onto the branch helix is highlighted in magenta.

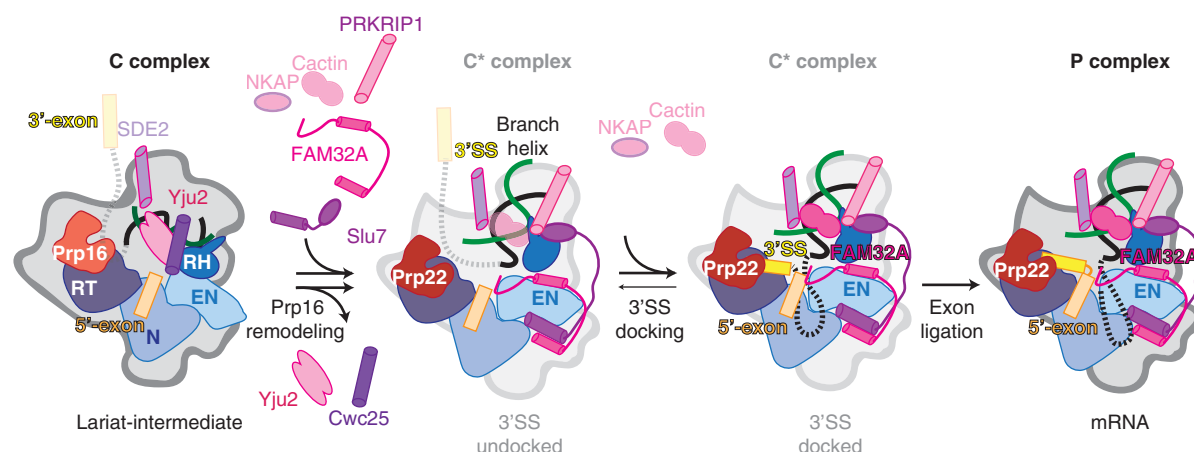


Fig. 5. Model for the action of exon ligation factors in metazoans. After Prp16 dissociates Cwc25 and Yju2 from C complex, Slu7, PRKRIP1, and FAM32A can bind the remodeled C* conformation. Cactin may bind before, or concomitantly with, docking of the 3'-exon at the catalytic core and associates more strongly upon 3'SS docking. SDE2 is likely present already in the C complex, as it interacts with the NTC, remains bound throughout the catalytic stage, and promotes Cactin binding after remodeling by Prp16. FAM32A binds the 5'-exon and likely stabilizes docking of the 3'SS onto the 5'SS and BP adenosine.

stabilizes the branch helix in the yeast P complex (fig. S10B) but which dissociates during the C to C* transition in humans (Fig. 5) (7).

In yeast, docking of the 3'SS is stabilized by Prp18, which abuts the 3'SS and guides Slu7 binding to Prp8. Indeed, in a subset of the yeast P-complex particles lacking Prp18 and Slu7 (7), the 3'SS is not stably docked in the active site and the branch helix shows weaker density, suggesting that the branch helix is mobile (7). By contrast, Prp18 was not detected by mass spectrometric analysis of the human C* and P complex spliceosome assembled on MINX pre-mRNAs (20, 26, 27), and Prp18 is absent in the cryo-EM structure of the human C* complex (22). The human P-complex structure presented here also lacks Prp18 (fig. S10, A and B, and table S3).

Notably, FAM32A penetrates into the active site of the P-complex spliceosome assembled on MINX pre-mRNA and promotes 3'SS docking, thus partly substituting for Prp18. In contrast, depletion of Prp18 from HeLa extracts abolishes exon ligation of β -globin pre-mRNA (32), raising the intriguing possibility that Prp18 promotes splicing of a subset of human transcripts, acting as in yeast. Indeed, in *S. pombe*, genetic depletion of Prp18 abolishes splicing in an intron-specific manner (33). Docking of the yeast Prp18 structure onto our human P complex indicates that Prp18 binding can be accommodated while FAM32A is bound in the active site of the human P complex (fig. S10, C and D). Hence, both Prp18 and FAM32A could influence alternative splicing of specific pre-mRNAs at the exon ligation stage. Consistent with this idea, Slu7 has been shown to influence selection of competing 3'SS by regulating docking of the 3'SS at the P-complex stage (34). Intriguingly, Slu7 does not closely approach the active site in our human P complex but binds FAM32A, which enters the active site. Thus, FAM32A could be responsible, at least in part, for the effects of Slu7 on 3'SS selection. Therefore, several exon ligation factors could modulate 3'SS choice during the catalytic stage.

Our P-complex structure highlights how in mammals specific proteins regulate a conserved mechanism for 3'SS recognition, and it also provides a framework to expand mechanistic studies of the human spliceosome to different cell types and different metabolic or developmental states.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/710/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S10
Tables S1 to S3
References (35–51)
PyMOL session

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REPORT

SURFACE SCIENCE

Density fluctuations as door-opener for diffusion on crowded surfaces

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How particles can move on a catalyst surface that, under the conditions of an industrial process, is highly covered by adsorbates and where most adsorption sites are occupied has remained an open question. We have studied the diffusion of O atoms on a fully CO-covered Ru(0001) surface by means of high-speed/variable-temperature scanning tunneling microscopy combined with density functional theory calculations. Atomically resolved trajectories show a surprisingly fast diffusion of the O atoms, almost as fast as on the clean surface. This finding can be explained by a “door-opening” mechanism in which local density fluctuations in the CO layer intermittently create diffusion pathways on which the O atoms can move with low activation energy.

We investigated the question of how atoms can move on a “crowded surface,” the situation on solid catalysts under operation conditions. Surface diffusion, which determines the mixing of the reactant particles on the catalyst surface and their lateral transport—for example, to defects acting as “active sites”—is generally regarded as extremely fast. This assumption is based on the low hopping barriers that the adsorbed particles can easily overcome at the applied elevated temperatures (*1*). Surface diffusion is thus usually not even included in the kinetics of catalytic reactions.

On the other hand, at the high pressures of industrial processes, catalyst surfaces can be highly covered by molecules adsorbing from the gas phase, reaction intermediates, and by-products. Whether the picture of an extremely mobile layer still holds for such crowded surfaces on which most adsorption sites are occupied is an open question. Measurements of macroscopic surface diffusion coefficients usually show a decrease with increasing coverages of the adsorbates (*2–6*) so that one may expect just the opposite: complete immobility at saturation. However, such macroscopic data are difficult to interpret with respect to atomic processes; simple site-blocking usually seems insufficient, and more complex effects—such as repulsive or attractive interactions between the particles, phase formation, and trapping at defects—play a role. For atomic diffusion mechanisms on crowded catalyst surfaces, one thus usually relies on kinetic Monte Carlo simulations (*7, 8*).

Some experimental, atomic-scale information of how particles may move when embedded in a close-packed two-dimensional (2D) layer has been obtained with scanning tunneling microscopy (STM). For In and Pd atoms embedded in the first layer of a Cu(100) surface, these “tracer atoms” moved by means of a vacancy mechanism (*9–11*), which is also the most prominent diffusion mechanism in 3D solids (*12*). The tracer atom can only jump to a neighboring lattice site when one of the mobile vacancies, which

exist in a solid at a finite temperature, comes near the tracer atom, so that it can exchange sites with the vacancy. For Cl atoms on an H-covered Si(100) surface, a direct exchange of the tracer atom with neighboring H atoms was suggested (*13*). Such a direct exchange mechanism has also been postulated for 3D solids but is usually not observed because of the high activation energies involved. On Cl- and Br-covered Cu(100) electrodes in an electrolyte solution, S tracer atoms were proposed to move by means of two other mechanisms known from 3D solids: a ring-exchange mechanism or a mechanism resembling the so-called interstitialcy mechanism (*14*). In both cases, the dynamics were strongly affected by the electrical potential and thus specific to the electrochemical environment.

Here, we show that in an adsorbate layer on a catalyst, which is chemically and structurally quite different from these systems, diffusion may follow a different mechanism. Using a combined high-speed/variable-temperature STM that achieves imaging rates of up to 50 frames s^{−1}, we investigated the dynamics of individual O atoms on a fully CO-covered Ru(0001) surface. This system was chosen because the CO oxidation on Pt group metals is a well-studied model for catalysis (*15*). Because (metallic) Ru is the least active of these metals (*16*), diffusion could be studied without competing CO₂ formation. By means of statistical analysis of movies that consist of a large number of STM images over an extended range of temperatures and complementary density functional theory (DFT) calculations, we obtained a complete description of the atomic mechanism. In a “door-opening” mechanism, local density

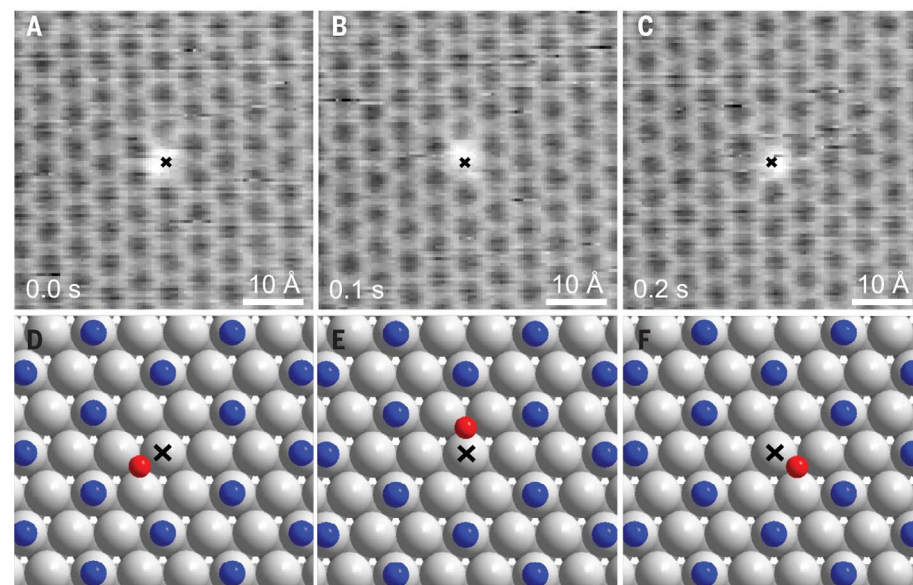


Fig. 1. Hopping of O in a CO cage. (A to C) Three consecutive STM images of the position of a single O atom embedded in the layer of CO molecules on Ru(0001). The bright dot is the O atom, and the hexagonal pattern is the $(\sqrt{3} \times \sqrt{3})R30^\circ$ structure of CO molecules (dark dots); the “x” marks a $(\sqrt{3} \times \sqrt{3})R30^\circ$ lattice point (300 K, 10 frames s^{−1}, tunneling voltage $V_t = -0.22$ V, tunneling current $I_t = 10$ nA). (D to F) Corresponding structure models. The O atom is indicated in red, the CO molecules in blue, and the Ru atoms in gray.

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fluctuations of the CO layer open paths on which the O atom can move with surprisingly low activation energy.

Three consecutive images from an STM movie taken at 300 K (movie S1) show a single O atom surrounded by CO molecules (Fig. 1, A to C). Close inspection revealed that the position of the O atom was not exactly on a lattice site of the CO structure but displaced somewhat to the left in Fig. 1A, up in Fig. 1B, and to the right in Fig. 1C. This asymmetry resulted from the different adsorption sites of O atoms and CO molecules (Fig. 1, D to F). The O atom occupied a hexagonal close-packed (hcp) site (a threefold hollow site with a Ru atom of the second layer underneath) (17, 18), whereas CO occupied a top site (19), so that the O atom is necessarily displaced with respect to the CO lattice site. Occupation of the face-centered cubic (fcc) site, the threefold site without a Ru atom underneath, was not observed for the O atoms, which is consistent with the calculated lower binding energy of O on this site (18). We ruled out that the lattice site marked by the “x” in Fig. 1 was occupied by a CO molecule because in such a configuration, CO and O would bind to the same Ru atom, which would result in a strongly repulsive interaction (20). The short time interval of 0.1 s between the movie frames already indicates that at 300 K, the O atom readily hopped between the three hcp positions in a “cage” defined by the neighboring CO molecules.

To record O atom trajectories over longer time periods, an automatic particle-tracking algorithm was developed that could identify the positions of the O atoms in the STM images and record position changes over several thousand images (supplementary materials, materials and methods, and movie S2). An example obtained from an experiment at 273 K is shown in Fig. 2. The trajectory displayed a striking sequence of equilateral triangles, with side lengths of ~ 2.5 Å, each triangle consisting of several lines and connected to a neighboring triangle by (mostly) a single line of the same length. An obvious explanation is that each triangle represents the three hcp positions, separated by the Ru lattice constant of 2.7 Å, which the O atom can occupy in a CO cage (Fig. 1). The trajectory can then be understood as a path where the O atom spends a large fraction of time hopping between the three positions within a cage but occasionally leaves it, after which it becomes trapped in a neighboring cage.

An example for such a cage-leaving event is shown in Fig. 3. The O atom, first located at the upper tip of the original triangular cage (Fig. 3A), appeared in the following image at the base of a neighboring triangular cage (Fig. 3B). Because the O atom, after this event, was again coordinated by six CO molecules (Fig. 3B), this process represents a site exchange between the O atom and a CO molecule (Fig. 3, C and D). The arrows shown in Fig. 3C are not meant to suggest that this process must be a direct exchange of the O atom with the marked CO molecule. The connected triangles in the O trajectories

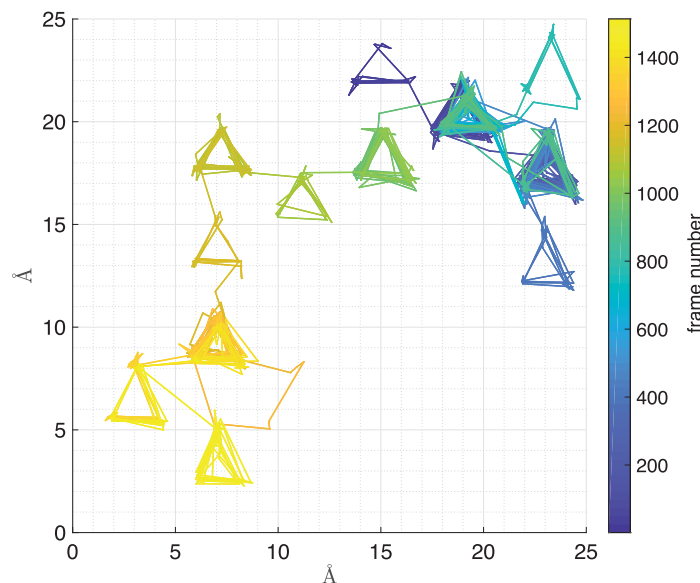


Fig. 2. Trajectory of an O atom through the CO layer on Ru(0001). The series was constructed from 1512 consecutive frames of an STM movie (273 K, 12 frames s^{-1} , $V_t = -0.7$ V, $I_t = 3$ nA). The positions of the O atom in the individual frames are connected by lines, and frame numbers are color-coded.

(Fig. 2) that represent the diffusion of the O atom on the CO-covered surface are thus explained by two processes, local hopping within the CO cages and exchanges with neighboring CO molecules.

To test the validity of this diffusion model and to extract hopping frequencies, we developed a statistical analysis method. The model assumes that the individual hopping events of the O atom are statistically independent of each other—a most certainly fulfilled assumption because the time between the hopping events is much longer than the 10^{-12} to 10^{-13} s time scale of the hopping events themselves. The hopping should then follow a Poisson distribution

$$\tilde{P}_{t_0}(n) = \frac{(\Gamma t_0)^n}{n!} e^{-\Gamma t_0} \quad (1)$$

where $\tilde{P}_{t_0}(n)$ is the probability that an O atom jumps n times during the time period t_0 . t_0 is the time for an STM image, and Γ is the hopping frequency, defined as $\Gamma = 1/\langle\tau\rangle$, where $\langle\tau\rangle$ is the mean time the particle spends on an adsorption site.

In the present case, the O atom can either hop with or without a site exchange with CO. For example, when the O atom is localized on the upper of the three hcp sites in the cage (Fig. 3E, inset), it can jump to one of the two lower hcp sites without exchanging sites with CO (“triangle jumps”) (Fig. 3E, green arrows). Alternatively, it can exchange sites with one of the three surrounding CO molecules from above (“exchange jumps”), along one of the four black arrows. Exchange jumps to the left and right are pairwise equivalent because of symmetry. Exchange jumps with the top CO and with one of the two lateral COs, although appearing different, are also equivalent: When the O atom has exchanged sites with the top CO (Fig. 3, C and D), the reverse of this process (Fig. 3, D to C) would involve a lateral CO. Because of microscopic reversibility, the back-and-forth processes must have the same rates, so that the exchange rates with the top and lateral COs must have the same rates. Therefore,

there are only two different types of processes that have to be considered, triangle and exchange jumps. $\tilde{P}_{t_0}(n_1, n_2)$ is then the probability that within the time period t_0 , the O atom jumps n_1 times within the cage and n_2 times through an exchange with CO. For the same reason as above, the two processes can be assumed to be statistically independent of each other, so that $\tilde{P}_{t_0}(n_1, n_2)$ is given by the product of two Poisson distributions

$$\tilde{P}_{t_0}(n_1, n_2) = \frac{(\Gamma_1 t_0)^{n_1}}{n_1!} e^{-\Gamma_1 t_0} \cdot \frac{(\Gamma_2 t_0)^{n_2}}{n_2!} e^{-\Gamma_2 t_0} \quad (2)$$

Γ_1 and Γ_2 are the frequencies of the triangle and the exchange jumps, respectively. Because the STM does not see the individual jumps but only records the particle position in a frame with respect to the preceding frame, $\tilde{P}_{t_0}(n_1, n_2)$ is not a directly measurable quantity. What is measurable is the displacement distribution $P_{t_0}(x, y)$, the probability that a particle located at $x = 0$ and $y = 0$ in one frame is found on a site with displacement coordinates x and y in the following frame. $P_{t_0}(x, y)$ is related to $\tilde{P}_{t_0}(n_1, n_2)$ by

$$P_{t_0}(x, y) = \sum_{n_1=0}^{\infty} \sum_{n_2=0}^{\infty} \tilde{P}_{t_0}(n_1, n_2) \cdot w_{n_1, n_2}(x, y) \quad (3)$$

$w_{n_1, n_2}(x, y)$ is the probability that the O atom can travel from $x = 0, y = 0$ to the coordinates x and y by a given combination of n_1 triangle jumps and n_2 exchange jumps. For example, for the starting configuration of Fig. 3E, inset, $w_{n_1, n_2}(x, y)$ to land on one of the two other triangle positions by the combination $n_1 = 1, n_2 = 0$ is 1/2 each. For general n_1 and n_2 combinations, the w_{n_1, n_2} values are obtained with recursion by using the geometry of the O/CO configuration, assuming that only jumps to neighboring hcp sites occur, for both processes (supplementary materials, materials and methods). Multiplication by $\tilde{P}_{t_0}(n_1, n_2)$ and adding overall n_1 and n_2 gives the theoretical displacement distribution $P_{t_0}(x, y)$. For the temperature

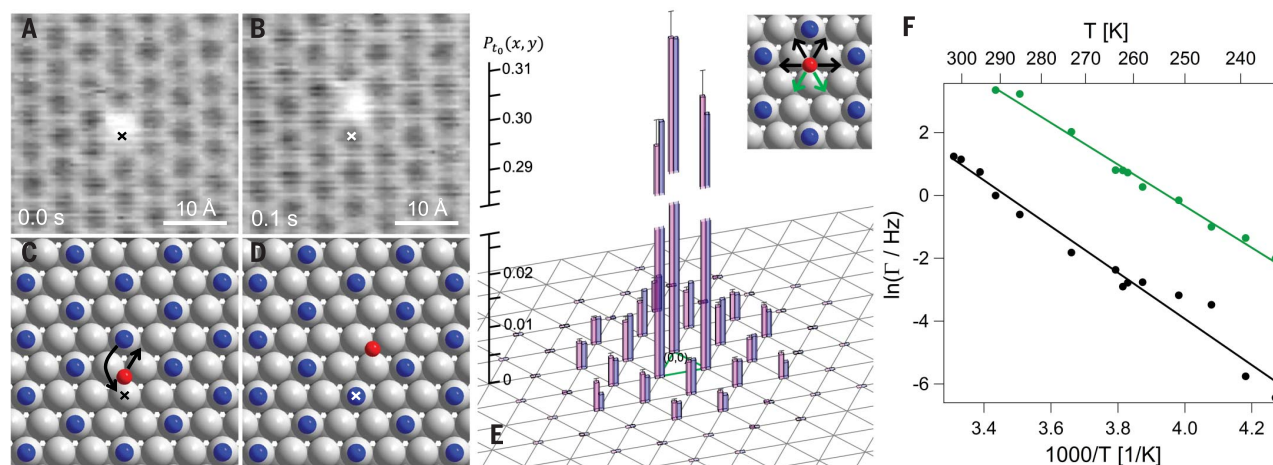


Fig. 3. Studies of O/CO exchange. (A and B) STM images of an exchange event between an O atom and a CO molecule (300 K, 10 frames s^{-1} , $V_t = -0.22$ V, $I_t = 10$ nA). (C and D) Corresponding structure models. The site marked by the "x" is the same in both frames. (E) Oxygen displacement distribution and hopping model. The magenta bars are the experimental displacement distribution $P_{t_0}(x,y)$, determined from 10,889

frames (291 K, 10 frames s^{-1}); the blue bars are the corresponding fit, and (0,0) is the starting position of the O atom. Green arrows in the model indicate jumps within the CO cage, and black arrows indicate exchange jumps with CO molecules. (F) Arrhenius plot of the two hopping frequencies. Green dots indicate jump rates Γ_1 within the triangles, and black dots indicate exchange rates Γ_2 with CO.

range investigated, the infinite sums could be terminated at $n_1 = 100$ and $n_2 = 10$. An analog equation has previously been derived for the analysis of field ion microscopy data, for the simpler case of one jump type in one dimension (27).

The experimental displacement distributions were determined, for each temperature, from several thousand to several tens of thousands successive frames by using trajectories such as that shown in Fig. 2. An example of the displacement distribution from measurements at 291 K is shown in Fig. 3E as the magenta bars. The high three central bars reflect the relatively long time the O atom spends within its cage, whereas the lower bars at larger distances are the result of the rarer exchanges with neighboring CO molecules. This distribution was least-square fitted with Eq. 3 (Fig. 3E, blue bars), where the only fitting parameters were the two hopping frequencies, Γ_1 and Γ_2 . The agreement of the fit with the experimental data is excellent (coefficient of determination $R^2 = 0.99994$). The frequencies obtained were $\Gamma_1 = 28.5 s^{-1}$ and $\Gamma_2 = 0.997 s^{-1}$. Similarly good agreement was obtained for the other temperatures.

The experimental distribution was well reproduced by Eq. 3, meaning that the two-process model provides a good description of O atom diffusion on the CO-crowded surface. A vacancy mechanism—the main mechanism in 3D solids, and also observed for atoms embedded in metal surfaces—can be ruled out. Vacancy diffusion displays characteristic spatial correlations of successive jumps resulting from the high probability after exchange with a vacancy that the tracer atom jumps back to the vacancy that is now located on the opposite side (9–11). Such correlations are absent here. However, the mechanism obtained here is also not a simple random walk. For example, for the O atom on the upper hcp site (Fig. 3E, inset) to exchange sites with one

of the CO molecules in the lower half, it first must jump to one of the two lower hcp positions. The resulting displacement is not independent of the order of the two jump types. This effect is fully implemented in the statistical model.

Displacement distributions were analyzed for several temperatures between 234 and 303 K. At temperatures < 234 K, the O atom jumped too rarely—a few times on the time scale of 1 hour—to give statistically relevant data. At higher temperatures, the rapid oscillations of the O atom in the CO cage prevented accurate determination of its position. Nevertheless, the frequencies measured in the accessible temperature range (table S1) span five orders of magnitude (very high for dynamic STM experiments), a result of the combined high imaging rate and low thermal drift enabling long observation times. The results for Γ_1 and Γ_2 plotted in Fig. 3F as functions of temperature gave straight lines on the Arrhenius plot for both processes over the entire temperature range. The activation energies obtained were $E_1^* = 0.57 \pm 0.02$ eV for the jumps in the triangles and $E_2^* = 0.63 \pm 0.03$ eV for the exchanges with CO. The preexponential factors, $\Gamma_1^0 = 10^{11.4 \pm 0.4} s^{-1}$ and $\Gamma_2^0 = 10^{11.1 \pm 0.7} s^{-1}$, were only marginally lower than the expected 10^{12} to $10^{13} s^{-1}$ range.

The two jump processes identified may consist of several elementary steps. The STM images show the particles in their stable positions, and metastable intermediate positions occupied by the O atom or the CO molecules may be too short-lived to be observable. Thus, in order to obtain further insights, we used DFT to calculate the energy landscape in which the jump processes take place using a technical setup (supplementary materials, materials and methods) that has been shown to be well suited for the CO/Ru(0001) system (22, 23).

For the triangle jumps, the hcp position of the O atom is most stable, and the fcc position

is a 0.30 eV higher local minimum. The barrier for the jumps from the hcp to the fcc position is 0.56 eV, which is in very good agreement with the experimental value for E_1^* of 0.57 eV. Thus, the most likely path that the O atom takes within a cage is from the hcp site via the fcc site to the neighboring hcp site.

For the exchange jumps, three scenarios were investigated: A direct exchange in which the O atom and the CO molecule moved concertedly (Fig. 3C), a sequential mechanism in which the O atom moved first, and a sequential mechanism in which a CO molecule moved first. Concerted mechanisms did not give low-energy paths along which a CO molecule actually exchanged sites with the O atom. A direct concerted exchange intermediately forced O and CO into a strongly repulsive conformation that resembled a deformed CO_2 molecule (fig. S2). The activation energy was very high, close to 1.5 eV, a value also found for the CO oxidation on Ru(0001) (20). Thus, a concerted, direct exchange mechanism could be ruled out.

A sequential mechanism in which the O atom moved first is indicated in Fig. 4A as the black arrows. In the initial step, the O atom moved from its original position (hcp 1) to an fcc site near the rim of the CO cage and then to an hcp site on the rim between two CO molecules (hcp 2). The energy profile of this path (Fig. 4B, dotted black line) showed a high barrier for the first step, 0.98 eV, resulting from O and CO sharing one Ru atom and strongly repelling each other in the intermediate O_{fcc}/CO configuration. The second step, the transfer of the O atom to the hcp 2 site, had a lower barrier of 0.35 eV. Also, the subsequent rearrangements of several CO molecules, such as along the indicated orange and cyan arrows, had low barriers of ~ 0.30 eV and were fast. In the final configuration, a CO molecule occupied the central top site in the

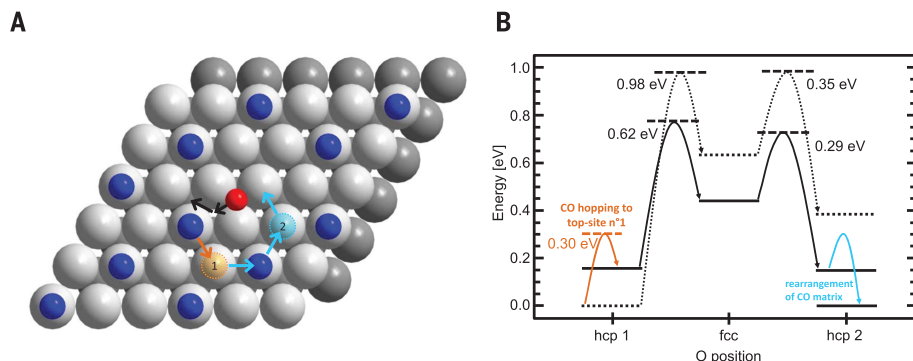


Fig. 4. Paths and energy diagram of the exchange jump of an O atom with CO. (A) Unit cell used for the DFT calculations. The black arrows indicate the path along which the O atom moves; the orange and blue arrows are paths taken by two CO molecules with possible intermediate positions 1 and 2. (B) Energy profile when the O atom moves first (dotted black line), and when a CO molecule moves first and then the O atom (solid black line).

cage originally occupied by the O atom. The net effect is a complete O/CO site exchange. However, the barrier of the first step is $>50\%$ higher than the experimental E_2^* value, which, given the high quality of the Arrhenius plot, we consider a substantial difference. Thus, we also rule out this mechanism.

In the sequential mechanism in which a CO molecule moved first, the initializing step could, for example, be a move of a CO to the top position 1 (Fig. 4A, orange arrow). The related barrier is 0.30 eV (Fig. 4B, orange line), and the energy of the O/CO configuration increased by 0.16 eV (Fig. 4B, solid black line). The same barriers and energy shifts were found when the CO initially moved to one of the other neighboring top sites. The increase in energy was caused by the mutual repulsions of the CO molecules on adjacent top sites, but this effect was relatively small. This repulsion also led to an outward tilt of the CO axes in the range of 5° to 9° , depending on the particular configuration. When, in the following step, the O atom moved to the indicated fcc site (Fig. 4A, black arrow), the barrier was considerably reduced to 0.62 eV (Fig. 4B, solid black line). The subsequent transfer of the O atom to hcp site 2 only had a barrier of 0.29 eV, and the consecutive rearrangements of the CO molecules, such as along the blue arrows in Fig. 4A, were again fast. Once the O atom moved to the hcp 2 site, it was much more likely for the CO molecules to rearrange than for the O atom to jump back. If we assume that the initial CO shift to a neighboring top site is a fast preequilibrium, the activation energy for the full process is the sum of the initial energy shift of 0.16 eV and the barrier for the O atom to jump to the fcc site of 0.62 eV, giving 0.78 eV. This value is the lowest from all mechanisms tested, and it was in reasonable agreement with the experimental E_2^* value of 0.63 eV.

The sequential mechanism in which a CO molecule moved first followed by the movement of the O atom described the STM data best. It resembles the ring-exchange mechanism in 3D solids, in which the tracer atom and several matrix atoms move in a ringlike fashion (12), but the

classical ring exchange is a concerted mechanism. We ruled out a concerted movement here because, in this case, the barriers of all elementary steps would add, giving unreasonably high activation energies. The mechanism is sequential, and it is better described as a local density fluctuation of the CO layer that occasionally opens a door, allowing the O atom to escape from its cage. Once the O atom has escaped, the locally disordered CO molecules quickly reorder, closing the door and preventing the O atom from immediately jumping back, which completes the exchange.

Such a diffusion mechanism is not known in 3D solids and has also not been reported for the diffusion of particles in close-packed surfaces. That such a mechanism occurred here can be understood by the CO molecules—despite forming an ordered overlayer—not being very densely packed, so that density fluctuations could occur with low activation energy. The mechanism is efficient, allowing the O atom to move at a surprising speed. For example, the frequency of the exchange jumps at 300 K of 4.7 s^{-1} [normalized by 6/4 to account for the reduced number of four directions in which the O atom can exchange with CO (Fig. 3E, inset)] is only by a factor of 3.5 lower than the hopping frequency of 16.6 s^{-1} of an O atom on the empty Ru surface (only measured at room temperature, so that no barrier is available) (24). The O atom could thus travel on the crowded surface almost as fast as on the empty one. Additional effects of a weakened O–Ru bond by the coadsorbed CO have been investigated but played a minor role (supplementary materials).

We propose that the door-opening mechanism may play a general role for diffusion on catalyst surfaces. Strongly bound particles such as O, N, or C atoms are intermediates in many catalytic reactions, and the door-opening mechanism would allow them to diffuse rapidly even in a crowded layer of weakly bound coadsorbates. High surface mobility in catalytic reactions is relevant for a correct formulation of the kinetics. Kinetic equations are usually formulated with coverages as variables. Coverages represent

mean-field averages, which is reasonable as long as the distribution of the particles on the adsorption sites is random. Because in a catalytic process the random distribution is permanently disturbed by the reaction, this condition is only fulfilled if the surface mobility is high enough to randomize the particles on a time scale fast with respect to the reaction.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/715/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S3
Table S1
References (25–34)
Movies S1 and S2

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DEVICE TECHNOLOGY

Printed subthreshold organic transistors operating at high gain and ultralow power

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Overcoming the trade-offs among power consumption, fabrication cost, and signal amplification has been a long-standing issue for wearable electronics. We report a high-gain, fully inkjet-printed Schottky barrier organic thin-film transistor amplifier circuit. The transistor signal amplification efficiency is 38.2 siemens per ampere, which is near the theoretical thermionic limit, with an ultralow power consumption of <1 nanowatt. The use of a Schottky barrier for the source gave the transistor geometry-independent electrical characteristics and accommodated the large dimensional variation in inkjet-printed features. These transistors exhibited good reliability with negligible threshold-voltage shift. We demonstrated this capability with an ultralow-power high-gain amplifier for the detection of electrophysiological signals and showed a signal-to-noise ratio of >60 decibels and noise voltage of <0.3 microvolt per hertz^{1/2} at 100 hertz.

Organic thin-film transistors (OTFTs) have driven the development in low-cost, large-area electronics, including emerging application areas (1–9), such as wearable technologies. These applications require devices that can bend and stretch without affecting their electrical behavior (10, 11). Organic

semiconductors have been widely investigated for this application, but circuits usually require a large operating voltage, leading to high power consumption and unsuitability for battery-powered operation (12–14). The most challenging part of wearable electronics is the sensor interface, which is an analog application requiring

low-voltage, low-power circuits with high gain, very high input impedance, low noise (15), and simple, low-cost fabrication (16, 17).

To meet these requirements, we used an inkjet-printed circuit technology (18) with a subthreshold Schottky barrier OTFT (SB-OTFT) that operates near the off state. This approach has three main advantages (19). First, these transistors exhibit a steep subthreshold slope, which allows the use of a low operating voltage and leads to a high transconductance efficiency. Second, the current-voltage relation (I - V) characteristics are independent of the channel length for a broad range of device geometries. These characteristics are ideal for printed electronics, because the variation in the typical inkjet-printed feature size of ~ 40 μm can be as much as 10 μm (fig. S12). Third, the intrinsic gain of the SB-OTFT is large (e.g., >1000) and independent of channel length and electrical bias, with a V - I signal amplification efficiency approaching the theoretical limit of $q/k_B T$, where q is the elementary charge, k_B is Boltzmann's constant, and T is temperature.

Defect density must be minimized within the printed structure to ensure a good Schottky barrier contact at the source-semiconductor interface (19). The Schottky contact energy barrier for hole injection into the organic semiconductor is established by the difference between the work function of the metal and the highest occupied molecular orbit (HOMO) in the organic semiconductor (19–21). We used 2,7-diocetyl[1]benzothieno[3,2-b][1]benzothiophene (C8-BTBT)

Fig. 1. Device structure and electrical characteristics.

(A) Schematic cross section of the SB-OTFT. PS, polystyrene; PVC, polyvinyl cinnamate; PEN, polyethylene naphthalate. (B and C) Measured transfer characteristics (I_D versus V_{GS}) of a typical device (B) on a linear scale, indicating the threshold voltage (V_T), and (C) on a log scale, indicating the subthreshold slope (SS), dec, decade. (D and E) Statistical distributions of (D) SS and (E) V_T for 50 devices. The dashed lines indicate normal distributions. (F) Measured output characteristics (I_D versus V_{DS}) indicating the output resistance (r_o) of devices with different channel lengths (L) and showing a full overlap of the characteristics. The inset shows r_o versus L .

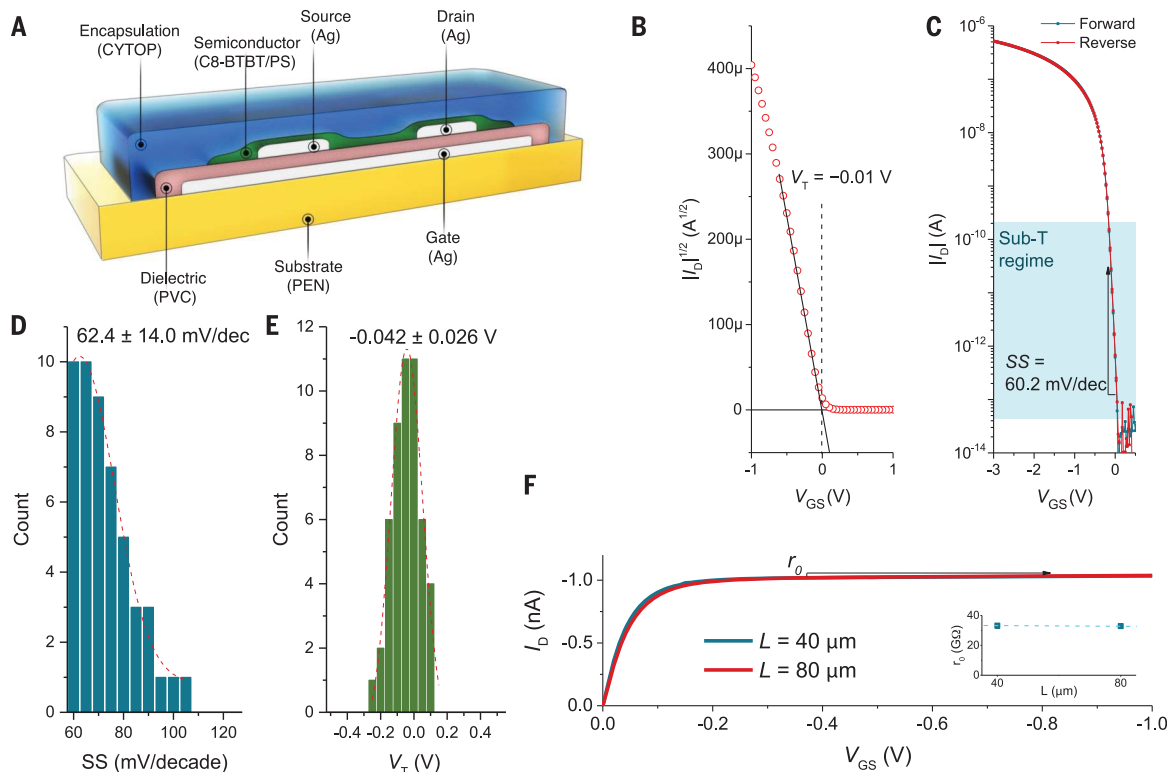
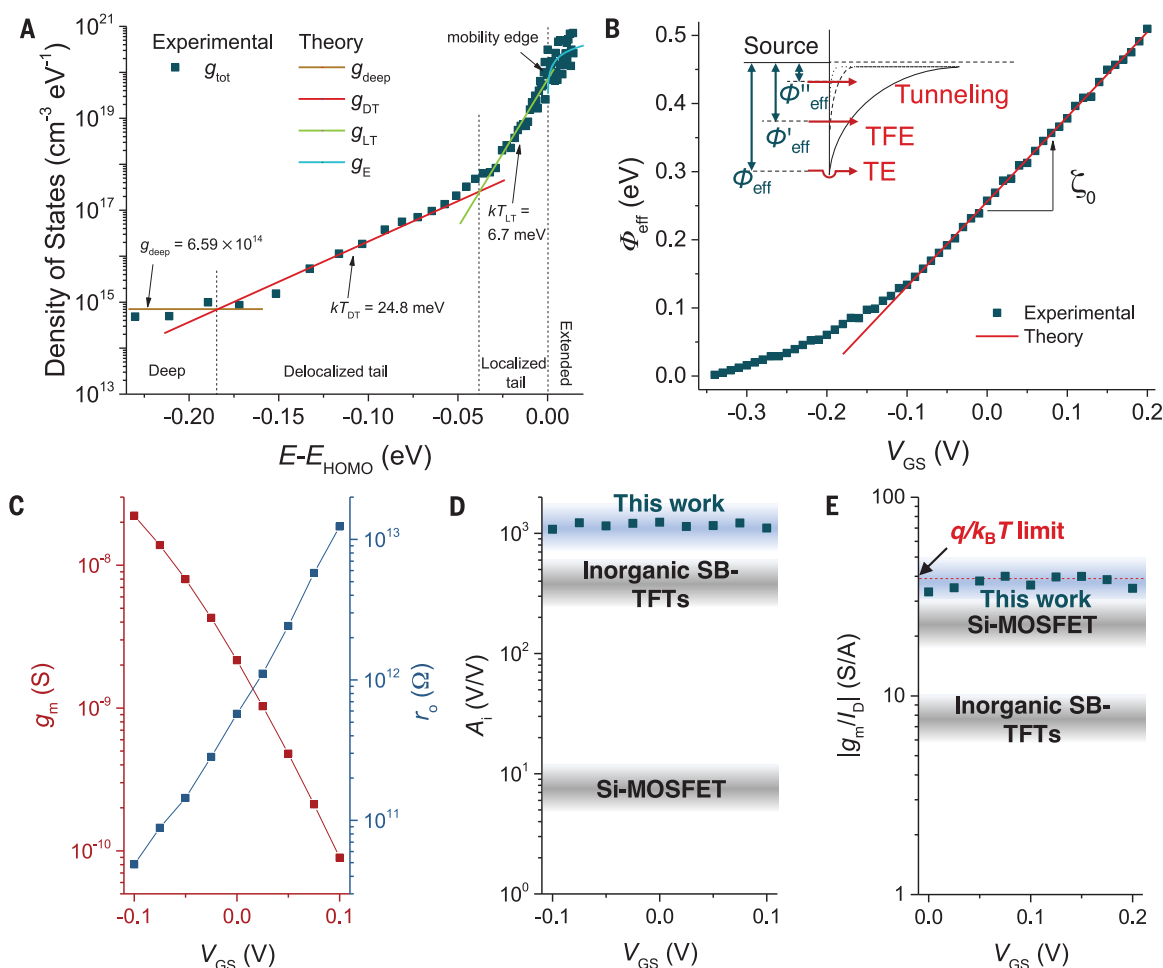


Fig. 2. Static parameters.

(A) DOS for a typical device, indicating four different regimes: deep states, delocalized-tail (DT) states, localized-tail (LT) states, and extended (E) states. The slopes in the DT and LT regimes indicate the characteristic energies ($k_B T_{DT}$ and $k_B T_{LT}$, respectively). g_{tot} , density of states.

(B) Effective Schottky barrier heights (Φ_{eff}) as a function of V_{GS} , indicating the gate modulation factor (ζ_0) for the Φ_{eff} lowering. (Inset) Schematic energy band diagram showing variation in effective Φ_{eff} and different charge-carrier injection processes. TFE, thermionic field emission; TE, thermionic emission. (C) Experimental values for g_m and r_o as a function of V_{GS} .

Ω , ohms. (D) Measured intrinsic gain (A_i) as a function of V_{GS} . Si-MOSFET, Si metal oxide semiconductor field-effect transistor. (E) Experimental values of transconductance efficiency (g_m/I_D) as a function of V_{GS} , reaching the theoretical thermionic limit of 38.7 S/A.



as the semiconductor (Fig. 1A), which exhibits fast growth (<1 min) of large crystals (>50 μ m) and has a lower HOMO level than pentacene or other derivatives (22) to yield a good Schottky barrier (>0.2 eV) (19, 21). Polyvinyl cinnamate was used as the dielectric layer to provide a smooth interface between the semiconductor and dielectric, thus minimizing carrier trapping and scattering (23). A fluoropolymer encapsulation layer (CYTOP) protected the device from environmental effects. Silver was used for the metal parts. All of these materials were formulated as inks with good jetting properties (fig. S1), and all of the fabrication steps for the individual SB-OTFTs and amplifier circuits reported here

were carried out by using a single inkjet printer tool.

The SB-OTFT demonstrated a near-zero threshold voltage ($V_T = -0.01$ V) (Fig. 1B), along with an ultra-steep subthreshold slope of $SS = 60.2$ mV per decade (Fig. 1C) that approached the theoretical thermionic limit (20):

$$SS_{\text{theoretical}} = \frac{\ln(10)v_{th}}{\text{decade}} = 59.6 \frac{\text{mV}}{\text{decade}} \text{ (at } T = 300 \text{ K)} \quad (1)$$

where $v_{th} = k_B T/q$ is thermal voltage. In addition, this steep SS is repeatable (fig. S3). The small V_T and steep SS resulted from the low trap density (20):

$$V_T = V_{T,\text{theoretical}} + \frac{Q_t}{C_i} \quad (2)$$

and

$$SS = SS_{\text{theoretical}} \left(1 + \frac{q^2 D_t}{C_i} \right) \quad (3)$$

where Q_t is the trap carrier density in coulombs per square centimeter, D_t is the defect trap density per electron volt and square centimeter, and C_i is the gate insulator capacitance in far-

ads per square centimeter. Q_t and D_t can be affected by defects in the semiconductor bulk (e.g., grain boundaries and stacking faults) and at the semiconductor-dielectric interface (e.g., interface roughness and atomic species or vacancies on dangling bonds). The relatively large semiconductor crystals in the thin-film transistor (TFT) channel (>50 μ m, providing good coverage over the channel) (fig. S2E) substantially reduce grain boundaries and stacking faults, compared to those in the amorphous or micropolycrystalline phases. The printed polymer dielectric layer was free of dangling bonds and provided a smooth semiconductor-dielectric interface (with roughness of 2.1 $\text{\AA}$) (fig. S2C). This was comparable to the roughness of the silicon-silicon dioxide interface in state-of-the-art complementary metal oxide semiconductor technologies. Thus, reducing the defect density to a very low level gives the best values for V_T and SS . Furthermore, the variation in these values between devices was much less than for other vacuum deposition-based TFT technologies (Fig. 1, D and E, and table S1). TFTs with a large C_i are effective in reducing V_T and SS (24) but lead to higher operating currents. Although this

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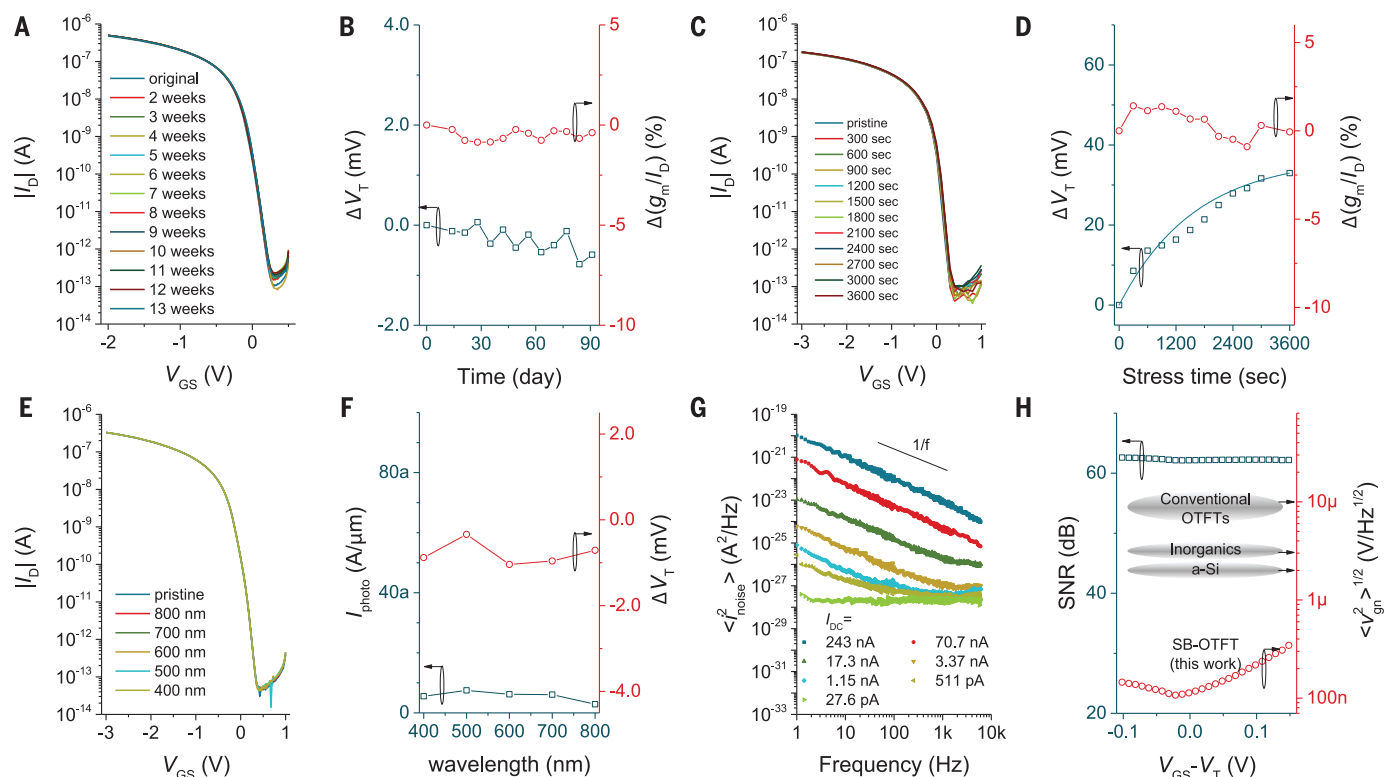


Fig. 3. Stability and reliability. (A) Measured transfer characteristics for a TFT in storage under ambient conditions for the times indicated and (B) change in absolute threshold voltage (ΔV_T) and change in relative transconductance efficiency [$\Delta(g_m/I_D)$] as a function of time. (C) Measured transfer characteristics under negative bias stress ($V_{GS} = V_{DS} = -3$ V) for the stress time indicated and (D) ΔV_T and $\Delta(g_m/I_D)$ as a function of stress time.

(E) Measured transfer characteristics under light exposure and (F) photo-current (I_{photo} in amperes per micrometer) and ΔV_T for different wavelengths (400 to 800 nm). (G) Measured SB-OTFT current noise under different direct current biases (I_{DC}). (H) SNRs in the near-threshold and subthreshold regimes and input-referred voltage noise density at 100 Hz. a-Si, amorphous silicon.

boosts the switching speed in logic circuits, it does not benefit the low-power, low-frequency operation of analog sensor interfaces.

We investigated the nature of the defect density and of the Schottky barrier through the density of states (DOS) (see Fig. 2A) and the effective Schottky barrier height (Φ_{eff}). These results suggest that the DOS comprises a small and constant background of deep states ($g_{deep} = 6.59 \times 10^{14} \text{ cm}^{-3} \text{ eV}^{-1}$, where g_{deep} is deep-state density), a broad spectrum of delocalized states with a characteristic energy of 24.8 meV near v_{th} , and a steeply rising number of localized tail states with a characteristic energy of 6.7 meV. In addition, because the DOS was dominated by extended states (according to $\sqrt{E - E_{HOMO}}$, where E is energy), there was a clear mobility edge for energies above the HOMO level (i.e., $E > E_{HOMO}$), characteristic of a small overall DOS. Because the semiconductor surface potential (ϕ_s) cannot be neglected in low-voltage TFTs, this term was included in the DOS calculation (eqs. S5 to S23).

The source-side Schottky barrier height (Φ_{eff}) decreased with increasing $-V_{GS}$, so that the drain current (I_{DS}) was modulated by the gate bias. Φ_{eff} could be extracted from temperature-dependent I - V measurements (fig. S6). In the subthreshold regime, $\Phi_{eff} = \zeta_0 V_{GS} + \Phi_{eff,0}$, where

ζ_0 is a coefficient that describes the modulation of Schottky barrier height by V_{GS} (19). Φ_{eff} showed a good initial Schottky barrier of ~ 0.51 eV and a high barrier-lowering factor of $\zeta_0 = 1.24$. This result suggests that charge-carrier injection was mainly by thermionic emission, with smaller contributions from thermionic field emission and tunneling (see the inset of Fig. 2B). Above a certain V_{GS} level (in the case shown at -0.34 V), barrier lowering saturated and the transistor behaved ohmically in the above-threshold regime. This change occurred when the source-side depletion width reached just a few nanometers and allowed charge carriers to tunnel through the Schottky barrier (fig. S5C) (20, 21). The small defect density and the presence of a good Schottky barrier in the subthreshold regime were prerequisites for high transconductance (mutual conductance g_m) and output resistance (r_o).

The near-zero V_T was important for low-power operation, whereas the ultrasteepest SS was important for high transconductance ($g_m = \partial I_{DS} / \partial V_{GS}$) and transconductance efficiency (g_m / I_{DS}) (eqs. S2 and S3). In addition, the SB-OTFT operation was channel-length independent with a large output resistance ($r_o = \partial V_{DS} / \partial I_{DS}$) (Fig. 1F), which was provided by the Schottky barrier at the source-semiconductor contact. Thus, the SB-OTFT could provide a high intrinsic

gain (defined as $A_i = g_m r_o$) (25) resulting from the high transconductance and output resistance.

Both the transconductance and output resistance had an exponential dependence, with an inverse proportionality, on $-V_{GS}$ because of the response of SB-TFTs in the subthreshold regime (Fig. 2C), as was also the case previously with an inorganic SB-TFT (19). In comparison with other TFT technologies, the SB-OTFT transconductance and output resistance are about 10 times as high at similar currents: $g_m = 3.8 \times 10^{-8} \text{ S}$ and $r_o = 3.2 \times 10^{10} \text{ ohms}$ at $I_{DS} = 1 \text{ nA}$ (1–6, 24, 26–28). The intrinsic gain A_i was determined from the theoretical expression (19)

$$A_i = \frac{SS_{theoretical}}{SS} n \exp\left(\frac{V_{eff}}{m_{th} v_{th}}\right),$$

where n is the ideality factor (here, $n = 1.6$). These devices showed a high and constant value for A_i of ~ 1100 in the subthreshold regime (Fig. 2D), which is much larger than that of the inorganic SB-TFT and Si metal oxide semiconductor field-effect transistor because of the ultrasteepest SS. More notably, g_m / I_{DS} for the SB-OTFT was $\sim 38.2 \text{ S/A}$, approaching the theoretical limit for TFT technologies of $q/k_B T$ (i.e., 38.7 S/A at $T = 300 \text{ K}$). The high g_m / I_{DS} (indicating a large g_m at low I_{DS}) was essential for an amplifier circuit to achieve high gain at low power. The SB-OTFT reported here

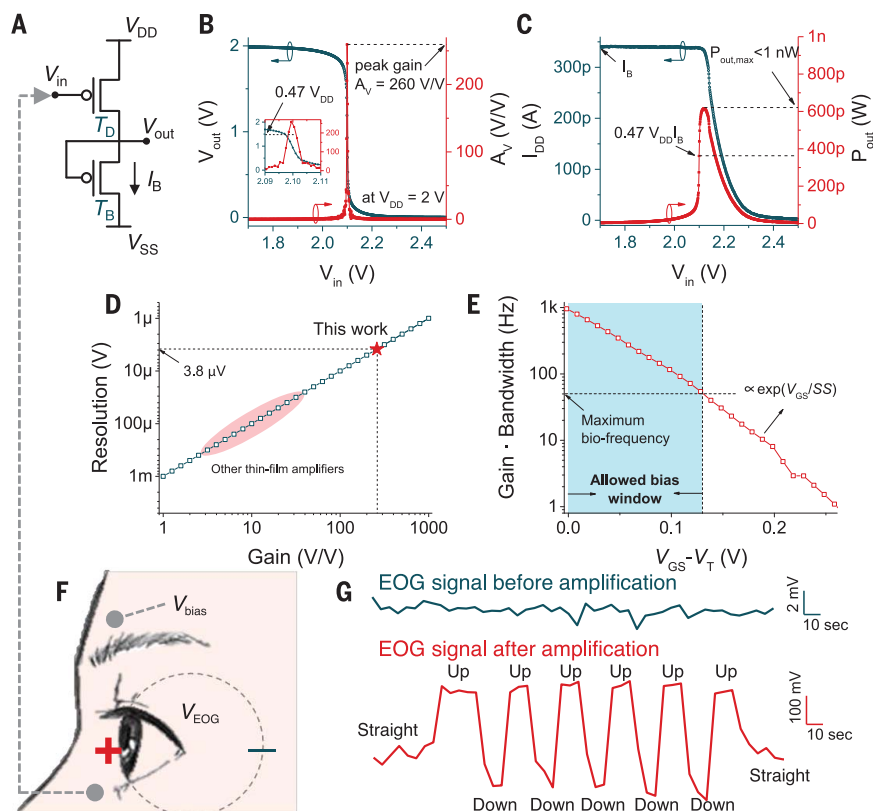


Fig. 4. Amplifier characteristics and demonstration of EOG detection. (A) Schematic circuit diagram of a common-source amplifier. V_{DD} , supply voltage; I_B , bias current. (B) Measured output voltage (V_{out}) and gain (A_V) as a function of input voltage (V_{in}). (C) Measured operating current (I_{DD}) and power (P_{out}) as functions of V_{in} . (D) Resolution of electrophysiological signal detection as a function of gain. (E) Gain-bandwidth product as a function of V_{GS} in the subthreshold regime. (F) Operating principle and circuit configuration for EOG amplification with the amplifier. (G) EOG signal obtained before and after amplification.

exhibited more efficient V - I signal amplification than the other reported devices (Fig. 2E).

The usability of inkjet-printed OTFTs is commonly limited by their short shelf life and operational instabilities (29, 30). However, when the transfer (I_D - V_{GS}) characteristics of representative SB-OTFTs were tested over a period of 3 months under ambient conditions, no appreciable changes were observed (Fig. 3A). The threshold voltage shift was <1 mV, and the transconductance efficiency changed by $<1\%$; thus, these SB-OTFTs were far superior under ambient environment operation and storage conditions than typical OTFTs, where these changes are generally >100 mV and $>20\%$, respectively (30, 31).

Similarly, the effect of electrical and illumination stress was very small (29–31). Electrical stress was applied under an on-state condition (i.e., $V_{GS} = V_{DS} = -3$ V), in which a conducting channel was formed and charge carriers were more likely to be trapped than in the nearly off-state condition. The transfer characteristics of the device before and after stress were almost identical (Fig. 3C). The threshold voltage shifted

by <30 mV with a characteristic decay time of $\sim 10^3$ s, and the transconductance efficiency changed by $<2\%$ (Fig. 3D). Because of the wide bandgap of C8-BTBT (fig. S7), the device demonstrated good light stability (Fig. 3E) under visible-light illumination stress (10 mW/cm 2), with a photocurrent of <10 aA/ μ m and a threshold voltage shift within 1 mV (Fig. 3F).

Noise ultimately limits the minimum detectable signal in any circuit, especially at the low frequencies of many electrophysiological signals (<100 Hz). The low-frequency noise response of SB-OTFTs showed both $1/f$ (where f is frequency) and white noise (Fig. 3G). As expected, these noise components were proportional to the current as I^2 and I , respectively (fig. S8, C and D, and eqs. S29 and 30). Thus, by operating in the subthreshold regime, the noise was reduced, giving rise to a signal-to-noise ratio (SNR) of 63 dB over the cutoff frequency of the TFT (Fig. 3H), which is sufficient for most low-frequency analog applications. The flicker noise coefficient is fabrication process dependent, and the value in our devices was 1.8×10^{-22} V 2 /F, which is one order of magnitude lower than

that found in typical amorphous Si- and metal oxide-based TFTs and two orders of magnitude lower than that in conventional OTFTs (table S1) (32). The root-mean-square noise voltage referred to the gate $\sqrt{\langle v_{gn}^2 \rangle}$ for all noise sources is <0.3 μ V/Hz $^{1/2}$ at 100 Hz (Fig. 3H), which is a few orders of magnitude lower than that of other TFT technologies for the same operating current.

We integrated amplifier circuits from pairs of SB-OTFTs in a common-source configuration, a drive transistor T_D and a bias transistor T_B (Fig. 4A). Because of the very high A_V of the SB-TFT, the amplifier demonstrated steep output voltage (V_{out}) characteristics and a voltage gain ($A_V = \partial V_{out} / \partial V_{in}$, where V_{in} is input voltage) of 260 V/V at the peak (Fig. 4B). Because transistor T_B operated in the subthreshold regime with a bias current $I_B = 342$ pA in the saturation regime, the power consumption was <1 nW (Fig. 4C). Compared to other TFT amplifiers, this high-gain amplifier enabled high resolution (<4 μ V) of electrophysiological signal detection (Fig. 4D). In addition, the gain-bandwidth product was scalable by gate bias. Given a maximum electrophysiological signal frequency of 50 Hz (33), the SB-OTFT had a relatively large allowed bias window for analog circuit design (0.13 V) compared to the variation of V_T .

Such an amplifier can be used to monitor human electro-oculogram (EOG) signals, which are essentially the corneo-retinal potentials (V_{EOG}) that exist across the front (positive) and back (negative) of the human eye (Fig. 4F), typically in the range from 0.2 to ~ 1.0 mV (34). This technique is useful for eye movement tracking, particularly in improving existing technologies that are bulky and costly and require high power (35). With a biasing electrode over the eyebrow and another electrode below the lower eyelid connecting to the amplifier input (Fig. 4, A and F), the V_{in} relation for the amplifier becomes

$$V_{in} = V_{bias} + \gamma V_{EOG}. \quad (4)$$

Here, γ is a coefficient that describes the direction of eye movement. In the configuration used, $\gamma < 0$ corresponds to an upward movement of the eyeball, whereas $\gamma > 0$ indicates the corresponding downward movement. Therefore, the amplifier output gives an amplitude of up to ~ 0.3 V (Fig. 4G and movie S1). The amplifier is also able to track horizontal eye movement (fig. S11). The amplified EOG signal with amplitudes of >0.2 V and SNRs of >60 dB has the potential to detect subtle eye movements for a better depiction of the virtual environment (e.g., depth-of-field rendering). Tracking eye movement is important in virtual and augmented reality (35). The ultralow power consumption of SB-OTFT-based circuits means that they can potentially operate from energy acquired from microharvesters (on the order of microjoules per cycle) (8), although from a complete system standpoint this would require low-power versions of signal-conditioning and transmission circuit stages.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/719/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S12
Tables S1 and S2
References (36–61)
Movie S1

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CERAMICS

Double-negative-index ceramic aerogels for thermal superinsulation

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Ceramic aerogels are attractive for thermal insulation but plagued by poor mechanical stability and degradation under thermal shock. In this study, we designed and synthesized hyperbolic architected ceramic aerogels with nanolayered double-pane walls with a negative Poisson's ratio (-0.25) and a negative linear thermal expansion coefficient (-1.8×10^{-6} per $^{\circ}\text{C}$). Our aerogels display robust mechanical and thermal stability and feature ultralow densities down to ~ 0.1 milligram per cubic centimeter, superelasticity up to 95%, and near-zero strength loss after sharp thermal shocks (275°C per second) or intense thermal stress at 1400°C , as well as ultralow thermal conductivity in vacuum [~ 2.4 milliwatts per meter-kelvin ($\text{mW}/\text{m}\cdot\text{K}$)] and in air (~ 20 $\text{mW}/\text{m}\cdot\text{K}$). This robust material system is ideal for thermal superinsulation under extreme conditions, such as those encountered by spacecraft.

Thermal insulation under extreme conditions, such as rapid temperature changes and long-term high-temperature exposure in aerospace and thermal power fields, requires exceptional stability and reliability for personal and property protection (1–3). Because of their low density, low thermal conductivity, and excellent fire and corrosion resistance (4, 5), ceramic aerogels are attractive candidates for thermal insulation. However, owing to their brittle nature and crystallization-induced pulverization behavior (6), ceramic aerogels often suffer from serious strength degradation and structural

collapse under large thermal gradients or extended high-temperature exposure. Examples of degradation that may result in catastrophic failure include structural cracks in silica aerogels (7), strength degradation for SiC aerogels (8), and volume shrinkage for alumina aerogels (9). Therefore, robust mechanical and thermal stability are the key roadblocks to using ceramic aerogels for reliable thermal insulation under extreme conditions.

Prior efforts to improve thermal stability primarily focused on overcoming brittleness by making flexible amorphous one-dimensional (1D) fibrous structures. Fiber-reinforced SiO_2 aerogels (10), SiO_2 nanofibrous aerogels (11, 12), SiC nanowire aerogels (8), alumina nanolattices (13), oxide ceramic (TiO_2 , ZrO_2 , and BaTiO_3) nanofiber sponges (14), and BN fibrous aerogels (15) have been developed with large, recoverable deformability (up to 80% compressive strain) derived from the elastic fibrous structures. However, owing to the large thermal expansion and pulverization behaviors of these ceramic materials and the weak point-linking pattern between 1D fibers, such fibrous ceramic aerogels still suffer from structural degradation under rapid thermal shocks or high temperatures. Moreover, the 1D fibrous building blocks lead to interconnected macroscale pores that cannot effectively mitigate solid conduction or convection in air. As a result, the thermal conductivities of fibrous ceramic aerogels are typically higher than that of stationary air [24 milliwatts per meter-kelvin ($\text{mW}/\text{m}\cdot\text{K}$)] (8–15).

In contrast, aerogels constructed from 2D nanosheets, such as graphene aerogels (16), feature durable face-to-face stacking interactions between 2D nanosheets to endow ultralarge deformability with up to 99% compression strain

(17, 18). Additionally, the face-to-face stacking between the 2D sheets also partitions the 3D aerogels into nearly isolated cells to effectively reduce the air conduction and convection, producing ultralow thermal conductivities below stationary air (19, 20). However, because of their easy oxidation and flammability, graphene aerogels are generally not stable in air at high temperatures ($>500^{\circ}\text{C}$). Porous ceramic structures synthesized by direct chemical reaction (21), elemental substitution (22), and template-assisted methods (23, 24) have attracted considerable interest, including template-assisted methods that produce 3D frameworks to replicate the template architecture. However, volume shrinkage and strength degradation of the resulting ceramic aerogels, which may be attributed to poor cross-linking between ceramic blocks and limited deformability of the typical templates, remain problematic (9, 11, 15, 23).

Materials with negative-index properties derived from specifically designed structures can substantially enhance performance metrics and allow for the development of distinctive attributes (25, 26). Mechanical metamaterials with a negative Poisson's ratio (NPR) have attracted considerable attention for their unusual mechanical enhancement for diverse applications, particularly in stringent environments such as aerospace and defense (27). By rationally manipulating the structure, materials with a NPR can deliver superior deformability and fracture toughness for overcoming the brittle nature of ceramic aerogels. Simultaneously engineering other negative indexes (28–32) may synergistically enhance additional physical properties, such as thermal stability.

Here, we report the design of a ceramic material with robust mechanical and thermal stability under extreme conditions. The ceramic has a double-paned metastructure with a NPR and a negative thermal expansion coefficient (NTEC). We used specially designed 3D graphene aerogel templates to synthesize hexagonal boron nitride aerogels (hBNAGs) and β silicon carbide aerogels (βSiCAGs) with excellent thermal and mechanical stabilities. The resulting hBNAGs exhibited ultralow density (~ 0.1 mg/cm^3), superelasticity (up to 95%), thermal superinsulation (~ 2.4 $\text{mW}/\text{m}\cdot\text{K}$ in vacuum and ~ 20 $\text{mW}/\text{m}\cdot\text{K}$ in air), and thermal stability under sharp thermal shocks ($\sim 275^{\circ}\text{C}/\text{s}$) and long-term high-temperature exposures (900°C in air and 1400°C in vacuum).

We designed a hierarchical porous structure with hyperbolic architecture to obtain a NPR (Fig. 1A). The subcells were designed with double-pane walls to reduce the wall thickness without compromising the mechanical strength and facilitate the out-of-plane vibration modes for NTEC (11, 13, 17). This metastructure design ensures widely distributed compressive strain under mechanical or thermal excitations (33) (fig. S1). To obtain the designed structure characteristics, we first produced the graphene aerogel templates using a modified hydrothermal reduction (MHR) and noncontact freeze drying (NCFD) technique (17, 18) and then prepared hBNAGs or βSiCAGs through a template-assisted

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chemical vapor deposition (CVD) process (Fig. 1B and figs. S2 to S4). For simplicity, we focus our discussion on hBNAGs. The resulting hBNAGs exhibit ultralow densities of less than 10 mg/cm^3 (17), with a lowest density of 0.1 mg/cm^3 , establishing them as a member of the group of super-light solid materials (3, 8, 11, 34–36) (Fig. 2, A and B, and fig. S5). We attribute the ultralow density of 0.1 mg/cm^3 to the highly porous structure with atomically thin cell walls (fig. S6).

We characterized the chemical composition and crystallinity of hBNAGs using x-ray photoelectron spectroscopy, Raman spectra, and x-ray diffraction (XRD) (fig. S7). The results from these characterizations reveal the high-crystallinity nature of hBN in the aerogel framework. In particular, the characteristic Raman and XRD peaks for hBN gradually narrow with increasing intensity on annealing at higher temperature, suggesting a systematic structural evolution from amorphous to polycrystalline BN. We also performed detailed structural characterizations of βSiCAGs (figs. S8 and S9). We investigated the morphology and structure of hBNAGs by scanning electron microscopy (SEM), Brunauer-Emmett-Teller measurement, spherical aberration-corrected transmission electron microscopy (TEM), and atomic force microscopy (AFM). The microstructure of the hBNAGs remained essentially the same as that of the graphene aerogel templates

(Fig. 2C and fig. S10), with the same facial-linking pattern between cell walls (fig. S11, and βSiCAGs in fig. S12). Thermal etching of graphene templates allowed formation of double-pane wall structures (Fig. 2D). The high bending stiffness of hBN prevented face-to-face collapse (37). We could tune the average gap size between the double-pane walls from a few to tens of nanometers (Fig. 2D and fig. S13) by controlling the wall thickness of graphene templates (17). The hBNAGs exhibited an ultrahigh porosity (99.99%) and a larger specific surface area ($1080 \text{ m}^2/\text{g}$) (fig. S14) than those reported for other ultralight materials ($\sim 800 \text{ m}^2/\text{g}$ for silica or carbon aerogels) (4, 16). The cell walls were made of highly crystalline hBN from planar-view TEM and selected-area electron diffraction studies (fig. S15). The cell walls consist of single or multi-layer well-ordered hBN with clearly resolved interlayer spacing of 0.33 nm (fig. S15), which we confirmed with AFM imaging (fig. S16). The wall thickness varied from 1 to 100 nm , depending on the precursor concentration in the CVD chamber.

We investigated the mechanical properties of the hBNAGs with uniaxial quasi-static compression (Fig. 3). We compressed the sample from 10 to 0.5 mm , a strain of 95%, and recovered the original configuration after pressure release (Fig.

3A and movie S1). The recoverable strain is higher than previously reported values for ceramic aerogels, which top out at 80% (8, 11–15, 38). We then demonstrated that the hBNAG sample can be repeatedly compressed at 90% strain for >100 cycles with little structural degradation (fig. S17 and movie S2). The Young's modulus is as high as 25 kPa for the first cycle with a slightly shrinking hysteresis loop during the next 20 cycles. The loop remains nearly unchanged up to the 100th cycle. The aerogel height remains nearly the same as the original value (residual strain < 4%), and the ultimate stress and Young's modulus gradually reach their equilibrium states with total decreases of 10 and 18%, respectively (Fig. 3B). We found similar superelastic behavior (strain up to 95%) in βSiCAGs (fig. S18), indicating that the templating method should be a general one for making elastic ceramics. Together, our aerogels show attractive mechanical properties when compared with ceramic aerogels with 1D fibrous structures (Fig. 3C) (8, 11–15, 38). For hBNAGs, the maximum strain is 95% compared with 80% for SiO_2 aerogels, the ultimate stress is up to 0.14 MPa compared with 0.03 MPa for Al_2O_3 aerogels, and the strength loss is 10% at $\epsilon_{100} = 90\%$ compared with 40% at $\epsilon = 50\%$ in amorphous BN aerogels.

Next, we investigated the dependence of the hBNAG deformation on the microscale wall

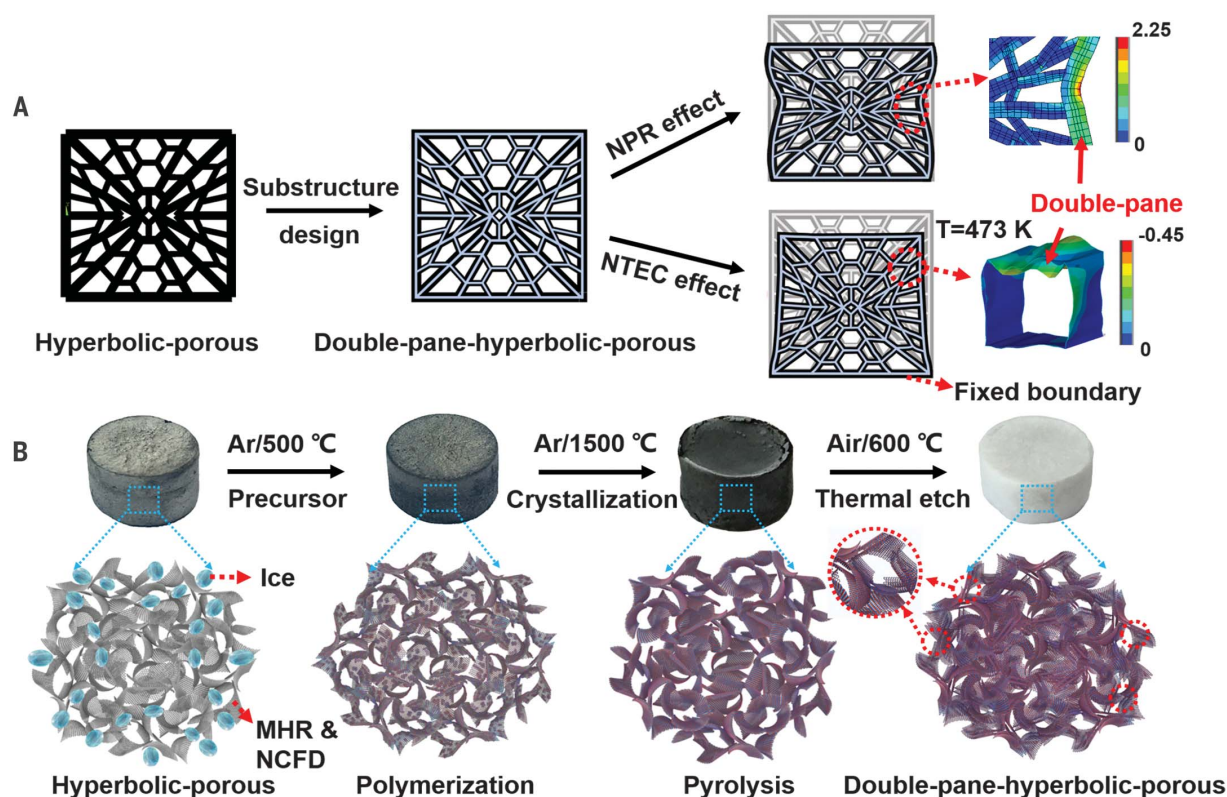


Fig. 1. Structure design and fabrication of the ceramic aerogel meta-material. (A) Illustration of the metastructure design of ceramic aerogels. The units of the colored scale bars are as follows: kilopascals for NPR and percentage (with strain zoomed by 30 times) for NTEC. (B) Illustration

of the CVD synthesis process of the double-paned hyperbolic ceramic aerogels. The NCFD technique is used to render hyperbolic structure in graphene aerogel templates by manipulating the ice crystal growth direction (17, 18).

thickness (Fig. 3D and fig. S19) and macroscale morphology to optimize the mechanical robustness of hBNAGs. The resilience of the walls determines their elastic behavior and depends on their thickness. Thin walls (<10 nm) have highly elastic (40

to 90% strain) behaviors due to their limited resilience. Increased wall thickness (up to 40 nm) changes the deformability to superelastic (90 to 95% strain). Further increasing the wall thickness (>60 nm) results in brittleness similar to other

bulk ceramic materials and a drop in ductility of 10% due to the confined bending deformation. Our observations are consistent with our molecular dynamics (MD) simulations (fig. S20) (33). A different way to look at this behavior is by plotting the relative Young's modulus (E/E_s) versus the relative density (ρ/ρ_s) as it scaled linearly as $E/E_s \sim (\rho/\rho_s)^{1.54}$ (Fig. 3E). This scaling corresponds well with the flexibility of hBNAGs within wall thicknesses of 1 to 40 nm. Samples with larger wall thickness (>100 nm) exhibit a larger $E/E_s \sim (\rho/\rho_s)$ trend of >2, which is similar to most existing rigid and brittle inorganic porous monoliths (11).

The theoretical structure design (fig. S1) and our previous studies (17, 18) show the mechanical benefits of having graphene aerogels with a NPR instead of positive or zero Poisson's ratios. The hyperbolic-patterned macrostructure facilitates the bending Poisson's effect (39) and triggers the oriented out-of-plane buckling of the cell walls and widely distributes compressive strain during the uniaxial compression of the samples (Fig. 3F, figs. S21 and S22, and movie S3). We demonstrated this with in situ SEM observations and analysis of strain mapping (fig. S23) (17, 33).

Thermally excited ripples could induce negative thermal expansion behavior in 2D nanolayered structures (40). Our double-pane structure design for the porous framework cell walls reduces the wall thickness for lower out-of-plane stiffness and releases by an additional degree of freedom to facilitate the out-of-plane vibration modes (11, 13, 17), leading to the contractions of the cell

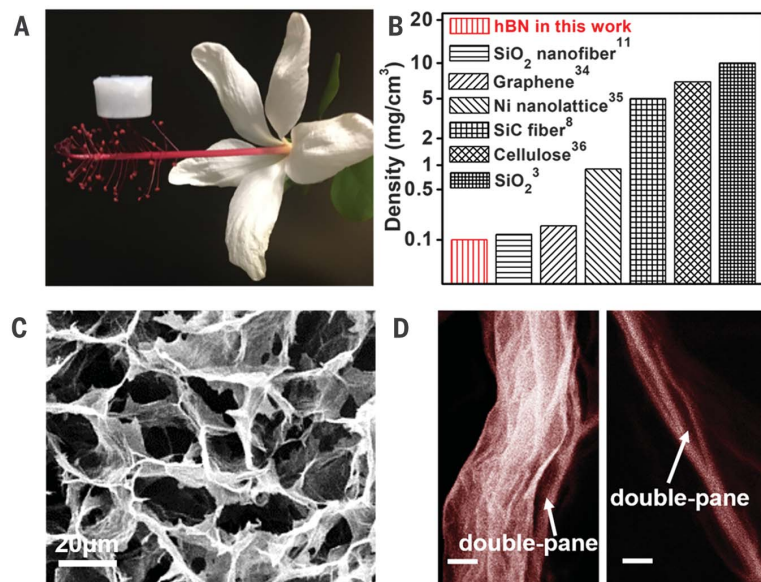


Fig. 2. Material characterization of hBNAGs. (A) An optical image showing an hBNAG sample resting on the stamen of a flower. All tests were done on ceramic aerogels with a density of 5 mg/cm³ unless otherwise noted. (B) The lightest hBNAG sample compared with other ultralight materials. The superscripted numbers indicate the corresponding referenced work. (C) SEM image of hBNAG. (D) SEM images of the double-pane wall structure of hBNAGs. Scale bars, 20 nm.

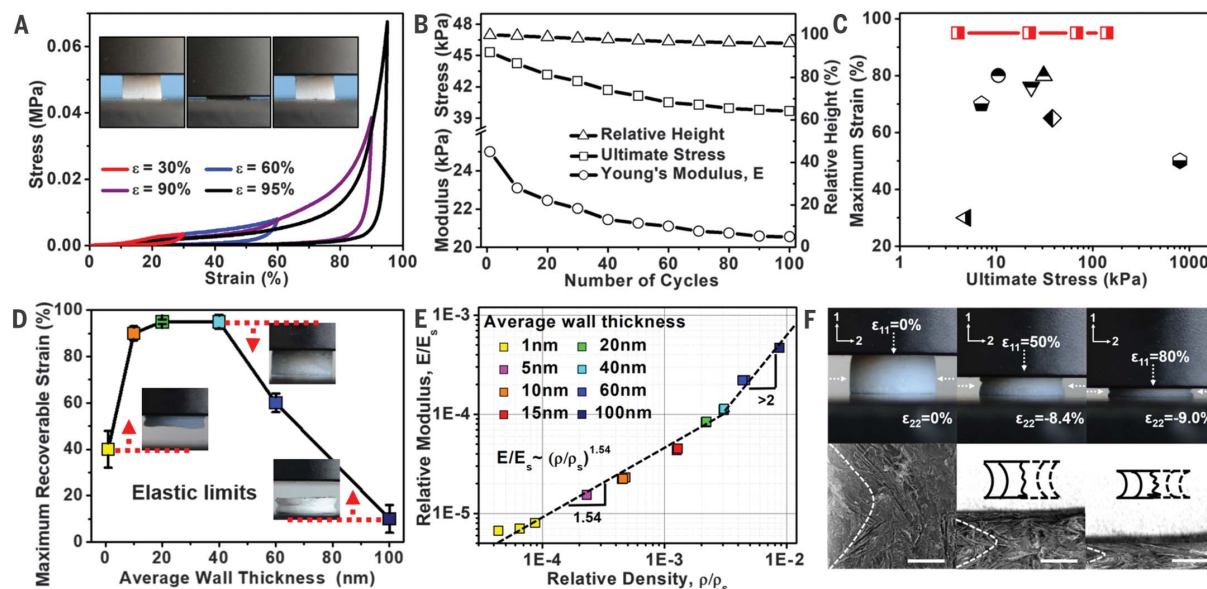


Fig. 3. Mechanical properties of hBNAGs. (A) Uniaxial compression of hBNAGs with repeatable strain (ϵ) up to 95%. (Inset) Experimental snapshots of one compression cycle. (B) The ultimate stress, Young's modulus, and relative height for 100 compression cycles. (C) The maximum strain and ultimate stress of the hBNAGs compared with other ceramic aerogels. Red square, hBN in this work; circle, SiO₂ with binder (12); right-side-up triangle, SiO₂ fiber (11); upside-down triangle, SiC fiber (8); pentagon, BN sheet (15); diamond, Al₂O₃ particle (38); hexagon, Al₂O₃ lattice

(13); sideways triangle, oxide ceramic fiber (14). (D) The maximum elastic deformability of hBNAGs as a function of the wall thickness. (Inset) Morphologies of hBNAGs with different wall thicknesses beyond their maximum elastic strains. (E) The relative modulus (defined as the measured Young's modulus, E , divided by the Young's modulus of the constituent bulk solid, E_s) of hBNAGs at relative densities. (F) Experimental snapshots of cross-sectional views and the corresponding SEM images of the NPR behavior of the hBNAGs under uniaxial compression. Scale bars, 1 mm.

walls in the frameworks. Meanwhile, the highly porous frameworks provide sufficient deformation space for these thermally excited ripples, and the double-pane structures further reduce the reciprocal constraints between adjacent cell walls. As a result, we realized a framework structure with NTEC [as shown in MD and finite element (FE) analysis in figs. S1, S24, and S25] (33). A SEM image (fig. S26) of the cell walls of hBNAGs suspended on a Cu grid shows small ripples at room temperature, which are likely formed during the deposition process. After annealing at 800°C and cooling, these small ripples evolved into larger ones (a large alteration in the ripple geometry, with apparently larger amplitudes and longer wavelengths), indicating the slack of the sample due to the TEC mismatch between the substrate and sample. This behavior was similar to what we observed in our previous work on graphene for the NTEC property (40). We measured the effective TEC of the hBNAGs (fig. S27); bulk hBN has a TEC of $40.5 \times 10^{-6}/^{\circ}\text{C}$ in the *c* direction (41), whereas the hBNAGs present an obvious linear NTEC of $-10.7 \times 10^{-6}/^{\circ}\text{C}$ below 80°C and $-1.8 \times 10^{-6}/^{\circ}\text{C}$ at higher temperature. β -SiCAGs also show linear NTEC behavior (fig. S28). Compared with the general tensile fracture induced by positive thermal expansion, the thermally excited compression stress in the NTEC case can be dissipated by the superelasticity of the hBNAGs, opening a pathway to enhanced thermal stability.

We further investigated the structural responses of hBNAGs under rapid thermal shocks. We designed a pneumatic thermal shock testing

system for the lightweight aerogel materials (fig. S29). By switching the pneumatic devices at the tube ends, we rapidly drove the ceramic aerogels back and forth between the fixed hot and cold sources. We swiftly heated the hBNAGs to 900°C and cooled to -198°C with a frequency up to 0.1 Hz and temperature variation speed up to 275°C/s (fig. S30 and movie S4) (33). We studied the mechanical strength and structure variation after 500 thermal shock cycles (Fig. 4A). The porous structure retained its original morphology (Fig. 4A, inset), and ultimate stress and maximum strain remained essentially unchanged. This indicated excellent structural stability and resistance to drastic temperature variations. Our NTEC material endured larger temperature swings (up to 1100°C) than silica aerogels ($<600^{\circ}\text{C}$) with even less strength degradation ($\sim 3\%$) (Fig. 4B and fig. S31) (33).

We also evaluated the effect of high-temperature stress and did not observe apparent weight loss below 900°C in air and 1500°C in Ar from the thermogravimetric analysis (fig. S32); the high crystallinity of the hBNAGs (fig. S7) prevented the weight loss. Above 900°C in air, we found oxidation-induced formation of B_2O_3 layers, resulting in an increase in weight. We kept the hBNAGs at 1400°C for 1 week and found no strength or volume loss (fig. S33). In comparison, previous SiO_2 , Al_2O_3 , and BN aerogels all degraded under long-term, high-temperature conditions (7–9, 23).

With excellent mechanical and thermal stability, the ultralight ceramic aerogels represent an attractive thermal insulation material family. In

particular, the distinctive double-pane pore structure suppresses air conduction and convection as well as reduces solid conduction contribution with its tortuous thermal pathway, leading to an ultralow thermal conductivity. For example, the resulting hBNAGs with a density of $5 \text{ mg}/\text{cm}^3$ exhibit a thermal conductivity (κ) of $2.4 \text{ mW}/\text{m}\cdot\text{K}$ at room temperature in vacuum conditions (pressure: $\sim 10^{-5}$ torr), which slightly increased to $8.19 \text{ mW}/\text{m}\cdot\text{K}$ at 500°C owing to the increased thermal radiation (Fig. 4C, figs. S34 to S36, and table S1) (33). In vacuum, the apparent thermal conductivity (κ_{total}) is the sum of radiation (κ_{rad}) and solid conduction (κ_{cond}) contributions. Taking advantage of the different temperature dependence of thermal radiation and conduction (33), we separated these two contributions and estimated κ_{cond} to be only $0.4 \text{ mW}/\text{m}\cdot\text{K}$, which is among the lowest for any freestanding material (2, 20, 33, 42, 43) (Fig. 4D and fig. S37). We ascribe this low matrix thermal conduction to three major factors, namely the ultralow density, the nanosized grains within the hBN sheets, and the double-pane wall structure. Density directly affects the actual conduction pathway and therefore κ_{cond} . However, because pristine multilayer hBN films have a high in-plane thermal conductivity around $400 \text{ W}/\text{m}\cdot\text{K}$, the low density ($\sim 0.2\%$ volume fraction) alone cannot explain the low κ_{cond} . The in-plane thermal transport within each sheet in the hBNAGs is highly suppressed by phonon scattering at grain boundaries because of the small grain size (50 to 100 nm). For few-layer hBN, calculations indicate this effect can reduce κ_{cond} by as much as 99% from the

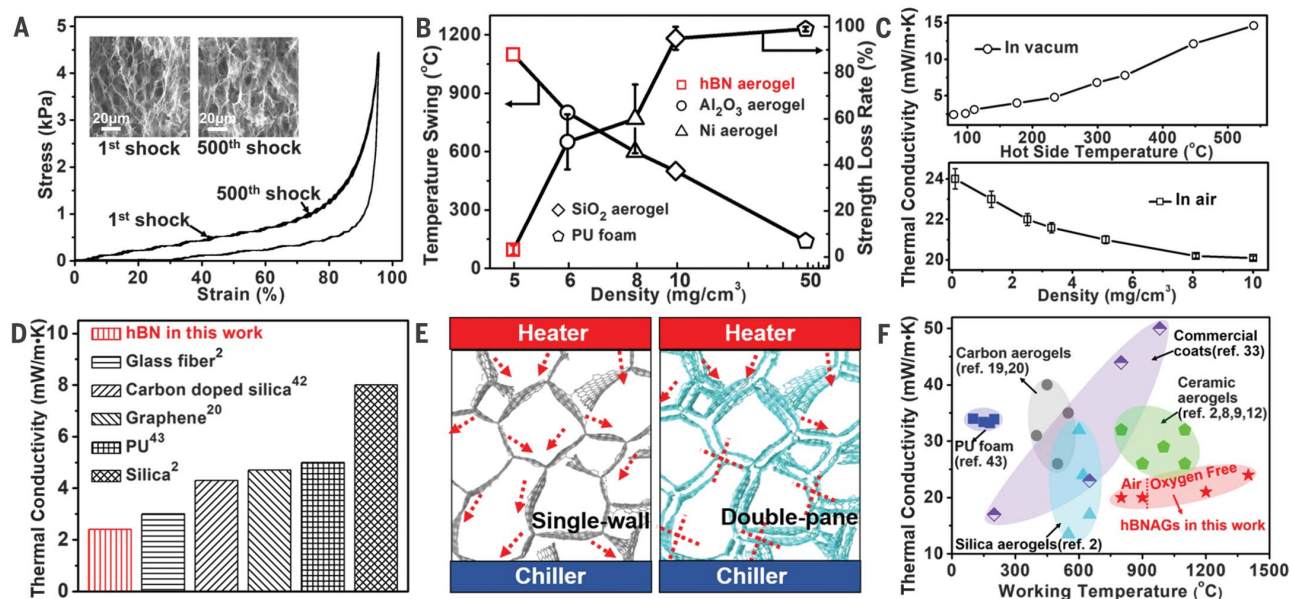


Fig. 4. Thermal stability and thermal insulation properties of hBNAGs.

(A) The strain and stress evolution after 500 cycles of sharp thermal shocks (275°C/s). (Inset) The SEM images of hBNAG frameworks after the first and last thermal shock tests. (B) The temperature differential and strength loss rate of hBNAGs for the thermal shocks compared with other aerogel-like materials. PU, polyurethane. (C) Thermal conductivity of hBNAGs in vacuum

(steady-state thermal measurement) and in air (transient thermal measurement). (D) The vacuum thermal conductivity of hBNAGs compared with other aerogel-like materials. The superscripted numbers indicate the corresponding referenced work. (E) The extra tortuous solid conduction path in double-paned hBNAGs. (F) Room temperature thermal conductivity in air versus working temperature for aerogel-like materials.

pristine value. Moreover, the interfaces connecting adjacent pores (Fig. 4E) create additional thermal resistance. For graphene aerogel with solid walls (19), this interface is a van der Waals bond, whereas for the hBNAGs with double-pane structure, because of the presence of the interface gap (~10 nm), heat transfer likely has to occur via near-field radiation (a much less effective process compared with van der Waals contact), thus substantially reducing κ_{cond} beyond the reach of conventional aerogels.

We also investigated the thermal conductivity at ambient conditions (Fig. 4C and figs. S38 to S41) (33). The hBNAGs with a density of ~0.1 mg/cm³ exhibited κ of 24 mW/m-K, which is similar to that of stationary air. We increased the hBNAG density to 10 mg/cm³, and κ decreased gradually to ~20 mW/m-K owing to the decreased pore size and the nanosized double-pane structures in the cell walls (36, 38). This thermal superinsulating performance is better than that of stationary air. We placed a 20-mm-thick hBNAG directly on an alcohol flame (~500°C) and then put a fresh flower on top of the hBNAG (fig. S42). The top surface of the hBNAG maintained a relatively low temperature of about 45°C after being held on the flame for 15 min, and the flower exhibited only slight withering.

Ceramic aerogels thus present a combination of low thermal conductivity and robust thermal stability that offers considerable advantage for thermal superinsulation exposed to extreme conditions (e.g., high temperature or sharp thermal shocks) (Fig. 4F) (33) under which polymeric (43) and carbonaceous (19) insulating materials could easily collapse or ignite, and the traditional ceramic aerogels, such as SiO₂, Al₂O₃, SiC, and BN (2, 4, 44), show poor mechanical stabilities.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/723/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S42
Table S1
References (45–62)
Movies S1 to S4

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QUANTUM OPTICS

Photoelectrical imaging and coherent spin-state readout of single nitrogen-vacancy centers in diamond

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Nitrogen-vacancy (NV) centers in diamond have become an important instrument for quantum sensing and quantum information science. However, the readout of NV spin state requires bulky optical setups, limiting fabrication of miniaturized compact devices for practical use. Here we realized photoelectrical detection of magnetic resonance as well as Rabi oscillations on a single-defect level. Furthermore, photoelectrical imaging of individual NV centers at room temperature was demonstrated, surpassing conventional optical readout methods by providing high imaging contrast and signal-to-noise ratio. These results pave the way toward fully integrated quantum diamond devices.

Electrical readout is a convenient way to measure the spin state of a qubit, and it has been successfully applied to semiconductor systems such as quantum dots (1, 2), phosphorous donors (3), and erbium ions (4) in silicon. However, all these examples require low temperature. In experiments with nitrogen-vacancy (NV) centers—which are prominent candidates for magnetic field sensing (5, 6), nanoscale

nuclear magnetic resonance (7), and quantum information processing (8, 9)—optical methods are used for defects visualization and spin-state readout. Recently, photoelectrical detection of magnetic resonance (PDMR) (10), photoelectrical readout of electron (11, 12) and nuclear (13) spins have been demonstrated on ensembles of NV centers under ambient conditions. However, the fundamentals of quantum technologies rest

on the coherent driving and readout of single qubits. Ensemble experiments demonstrated a strong background signal produced by nitrogen impurities that provide additional contribution to the photoinduced current. For this reason, the step toward single NV center detection has been a challenging task.

The idea of photoelectrical detection is based on intrinsic charge dynamics occurring under continuous light illumination (14, 15). The excited state of the negatively charged NV center (NV^-) lies close enough to the conduction band, so that the electron promoted to the excited state can either radiatively decay or undergo further excitation to the conduction band (Fig. 1A). There, it can freely travel under an applied electric field and finally be detected. However, photoionization alters the charge of the defect and thus its electronic level structure. The ground state of the neutral NV center (NV^0) is shifted toward the valence band, enabling recovery of the negative charge state. The only requirement for dynamical cycling between these two counterparts is that the photon excitation energy should be greater than the energy of the zero-phonon line of the NV^0 center (15). Within one cycle, two charge carriers are produced: one electron during ionization and one hole during NV^- recovery. This dynamical charge circulation enables the photoelectrical readout of NV centers, unlike other defects.

For our experiments, two types of diamond were used. The first type (represented by two ultrapure diamonds called sample a and sample b below) was undoped, ultrapure, chemical

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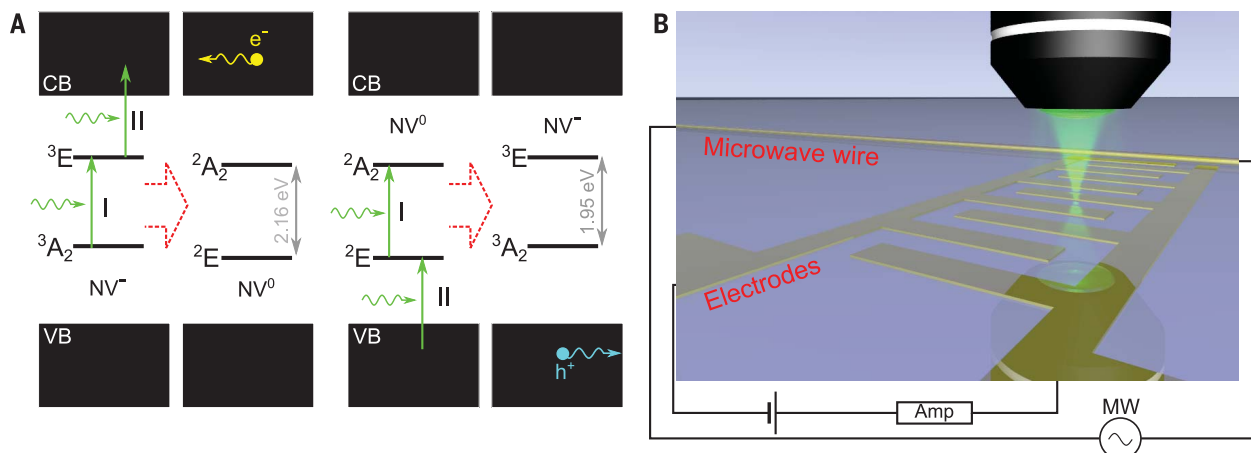


Fig. 1. Schematic representation of the experiment. (A) Simplified representation of the charge-carrier generation within one cycle of NV^- ionization and recovery. First, two photons produce one free electron in the conduction band (CB) and leave defects in the neutral charge state. Second, two photons convert the neutral charge state back to NV^- , while a free hole remains in the valence band (VB). I and II indicate

first and second transitions of the electrons. e and h represent electron and hole, respectively. (B) Configuration of the single-center photoelectrical imaging setup. Coplanar and electrodes deposited on the diamond surface are connected in series with a power supply and current amplifier (Amp). The microscope objective lens scans over the sample surface, while a microwave (MW) field can be applied via a 50- μm -thick wire placed near the electrodes.

vapor-deposited (CVD) diamond with a nitrogen concentration below 0.15 parts per billion and which contained only native NV centers. The second type was a diamond produced by nitrogen doping during chemical vapor-deposition growth (sample c). This sample contained engineered shallow NV centers, which is relevant for sensing applications. Subsequently, this diamond was polished under a small angle to form a gradient of shallow NV centers along the sample surface. Titanium electrodes coated with either gold or aluminum were produced on the surface by photolithography (see the supplementary materials for sample preparation). The distance from the NV centers to the electrodes varies from 1 to 20 μm .

We started by characterizing an area between the electrodes of the ultrapure sample a in confocal arrangement (Fig. 1B) and found a few sparse NV centers at a depth of $\sim 16 \mu\text{m}$. An example optical image is shown in Fig. 2A. The photon autocorrelation function was recorded on several defects. An antibunching dip below 0.5 at zero delay clearly indicates single defects (Fig. 2B). The same area was scanned by a focused laser beam while applying a voltage across the electrodes, and the electrical current flowing between electrodes was recorded as a function of illuminated point coordinates (Fig. 2C).

To characterize the photocurrent produced by a single center, we performed the following steps.

First, the voltage across the electrodes was set to the point at which current saturates. Further increase of the voltage is unreasonable owing to contact leakage (see the supplementary materials). Second, a series of scans with electrical readout were obtained for different values of laser power. In each image, the spot corresponding to the NV center is fitted by a Gaussian function (Fig. 2D) and the amplitude and background values extracted. They are plotted as a function of laser power in Fig. 2F. The photocurrent originating from a single NV center has a contribution from both types of charge carriers: electrons and holes. Under green excitation, their generation is a two-photon process

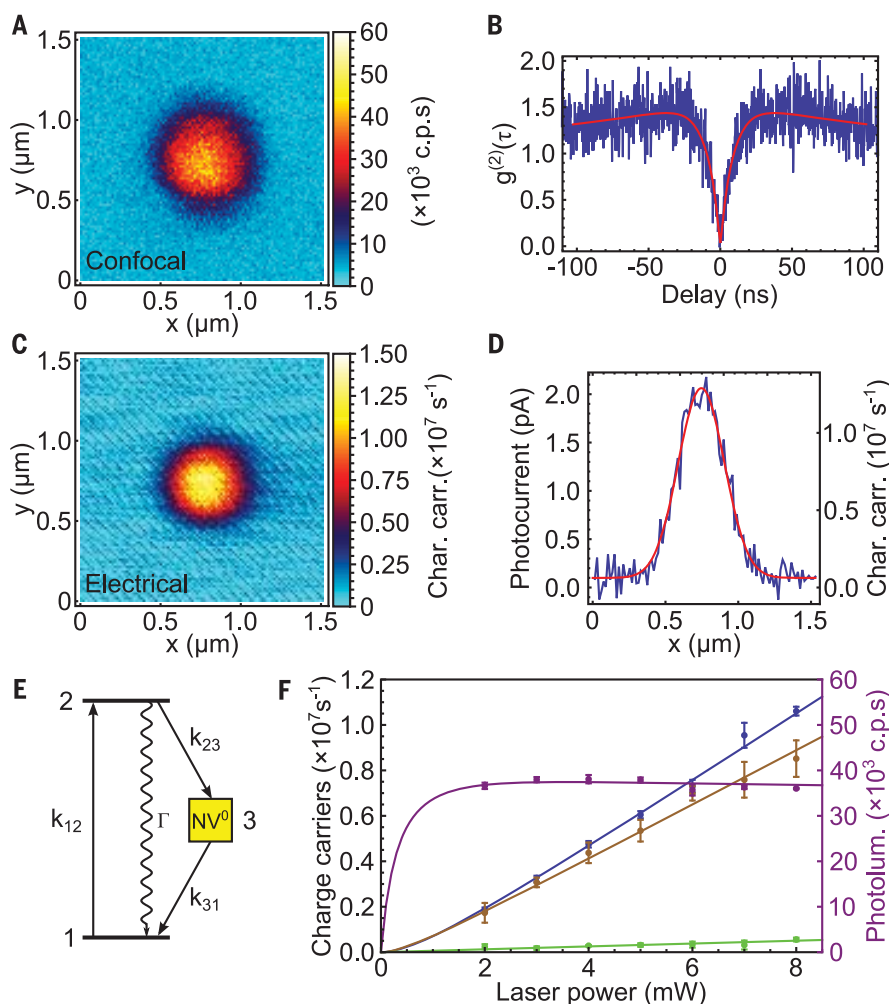


Fig. 2. Photoelectrical imaging. (A) Confocal image of the 1.5- μm -by-1.5- μm area between electrodes. (B) Photon autocorrelation function [$g^{(2)}(\tau)$] obtained from the spot in the image (A) indicates that a single NV center is addressed. (C) Photoelectrical image of the same area recorded at 9 mW of laser excitation power and applied voltage of 80 V. (D) Cross-section through the middle of the image (C), fitted with a Gaussian function. (E) Three-level model used to estimate the number of charge carriers generated under constant laser illumination. The model does not differentiate between the metastable state and another charge state. (F) Comparison of the number of charge carriers generated per second obtained from photocurrent measurements (blue data points) and from antibunching (brown data points). Both curves coincide well at low power. The deviation that occurs for increased powers results from the increasing fit error of antibunching curves. Green data points show background dependence. Purple data points represent the number of detected photons per second as a function of laser power. The solid purple curve is the fit to the data by the same model used for estimation of charge-carrier generation rate. The only free fit parameter is the photon detection efficiency (0.16% from the fit).

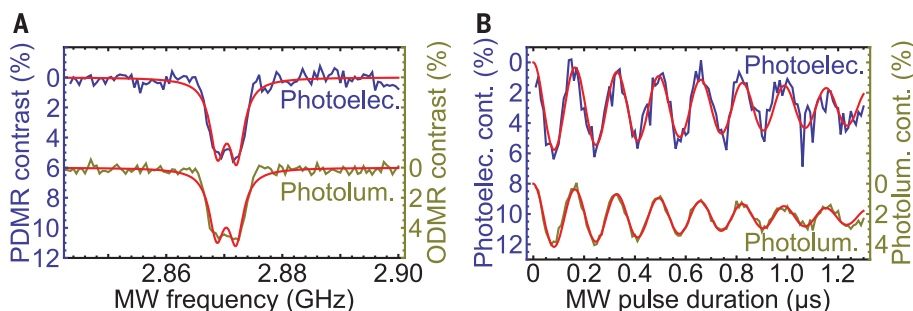


Fig. 3. Photoelectrical readout of a single electron spin. (A) Contrast of the PDMR (4.6%) and optically detected magnetic resonance (ODMR) (4.3%) signals simultaneously measured on a single NV center. The experimental data were corrected for a linear drift and were fitted using a double Lorentzian function (red line). (B) Contrast of the photoelectrically (6%) and optically (4%) detected Rabi oscillations. The red lines correspond to a fit of the experimental data.

with a scaling that can be described by the function $(I\beta)^2/(1 + \beta I)$, where I is the laser intensity and α and β are constants. At the low power limit, the curve has a quadratic growth, indicating a two-photon process. At higher powers, the curve becomes linear, because the first transition saturates and the population of the intermediate level changes weakly. The background photocurrent remains small for the entire range of measured powers. Its linear growth suggests that the background originates from other defects, such as substitutional nitrogen, in the diamond crystal.

To make a rough estimation of the charge-carrier generation rate, we used a simple method based on photon autocorrelation function measurements. The second-order autocorrelation function exhibits an antibunching dip at zero-delay time and bunching wings at finite delays. Whereas antibunching is a signature of a single photon source, bunching shoulders characterize the internal dynamics of the system and indicate the presence of a shelving state, where the population is trapped for some time. Previously, this trapping state was typically associated with metastable states of the NV⁻ center (16), although it was known that the bunching time is power-dependent (17). This fact contradicts the non-radiative nature of the relaxation dynamics from the excited state to the metastable state. Photoionization is another mechanism responsible for the excited-state depopulation, which, indeed, depends on the laser power and has not been taken into account previously. Hence, at low laser intensities, shelving to the metastable state defines bunching, whereas with an increase in the laser intensity, photoionization-induced “shelving” into the neutral charge state starts to play the primary role. This assumption is also supported by the power-dependent deshelving rate. Therefore, we can estimate the number of electrons and holes produced during illumination of the NV center using a simple three-level system depicted in Fig. 2E. Using this model, we fit the photon autocorrelation function to extract the rates of photoionization and negative-charge-state recovery (see the supplementary materials). The number of electrons (holes) generated per second is found as a product of the ionization k_{23} (recovery k_{31}) rate with ρ_2 (ρ_3)—the steady-state population of the level from which the transition occurs. The photocurrent is thus defined as $I_{pc} = (k_{23}\rho_2 + k_{31}\rho_3)e$, where e is the elementary charge of an electron. Comparison of the charge carrier's generation rate extracted from photocurrent measurements (blue) and from the photon autocorrelation (brown) is depicted in Fig. 2F. The data are in good agreement at low laser powers, whereas a small deviation is observed at higher powers. This mismatch arises from an increasing error for the rate extraction at high laser powers. This is due to narrowing of the antibunching dip and to the reduction of its contrast caused by timing jitter of the avalanche photodiodes. The purple data points in Fig. 2F show the number of extracted photons from the same NV center that reaches its maximum at about 3.8×10^4 counts

per second (c.p.s.) and then slightly decreases owing to reduction of the population ρ_2 caused by ionization. A fit to the data was done by $\eta\Gamma\rho_2$ (where Γ is the radiative decay rate of the excited state) with the parameters obtained from photon autocorrelation measurements, so that only the detection efficiency η was used as a variable fit parameter. Whereas the saturation

behavior fundamentally limits the number of detectable photons, linear scaling of the photocurrent allows production of more charge carriers for detection by a simple increase in the laser excitation power. The same center yields about 1.1×10^7 charge carriers per second at a laser power of 8 mW.

With the ability to detect and image the photocurrent resulting from the ionization of a single NV center, we demonstrate PDMR as well as electrical readout of a coherently prepared electron spin state. For this experiment, we used sample b, in which native single NV centers could be found at a depth of $\sim 2 \mu\text{m}$ below the diamond surface. To avoid limitations imposed by the high laser power onto the PDMR contrast (see the supplementary materials), not only Rabi but also PDMR measurements were performed in a pulsed mode. Figure 3 shows a comparison of the electrically read out magnetic resonance and Rabi oscillations of the NV center spin with simultaneously obtained optical signals. In the conditions considered here (see the supplementary materials), the contrast of the signals obtained by photoelectrical detection is comparable to or even exceeds the optical one.

To explore photoelectrical imaging, we utilized the diamond sample c with a variable density of NV centers situated within a depth of a few nanometers below the surface. Simultaneously obtained images of confocal and electrical scans are depicted in Fig. 4A. The area shows bright clusters of NV defects as well as single centers verified by antibunching measurements. Compared to electronic grade diamond, this sample presents a lower-lying layer of NV centers in the Ib substrate. The CVD layer also contains a higher concentration of nitrogen impurities than NV centers (conversion ratio of nitrogen into NV defects of only 100 to 1) (18). These different factors lead to a background photocurrent that constrains the imaging contrast. By performing measurements similar to those performed on sample a, we found that this background restricts the optimal laser power to between 2 and 4 mW, where the imaging contrast reaches its maximum of 98% (green data points in Fig. 4B). The contrast of electrical imaging always remains better than that of optical imaging. The latter does not exceed 62% (orange data points), owing to high background fluorescence from the NV layer beneath that cannot be completely discriminated by the pinhole. The background fluorescence also reduces the optical signal-to-noise ratio (SNR) (orange data points in Fig. 4C). Photoelectrical SNR (green data points) is limited by parasitic cross-talk, mainly 50-Hz technical noise of the electrical network, clearly visible in Fig. 4A as a set of periodical lines, which we could not completely suppress. The technical noise can be further improved by a proper design of the detection circuit, and the electron shot noise limit can ideally be reached.

The results presented here demonstrate the high potential of photoelectrical detection of coherently driven single NV centers, opening possibilities for simple and reliable implementation of diamond quantum devices that are

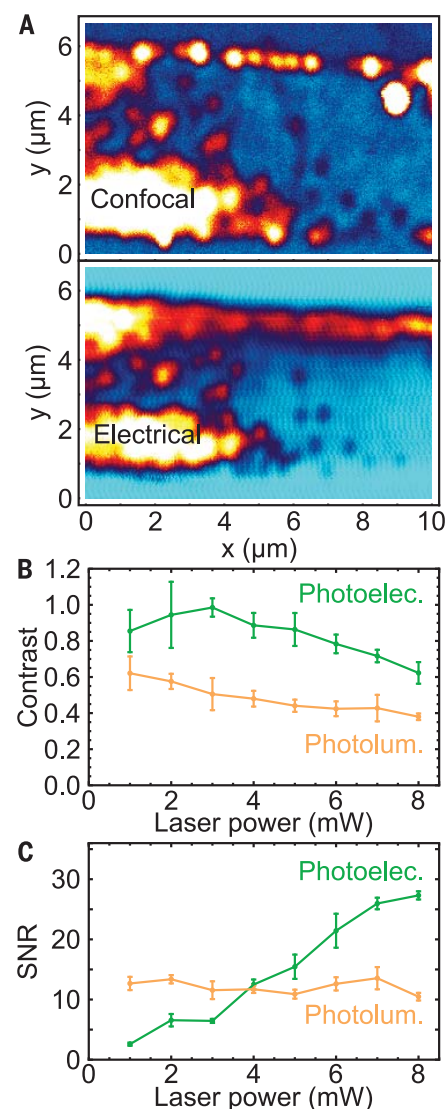


Fig. 4. Photoelectrical detection of engineered NV centers. (A) Confocal and photoelectrical images of the same area. (B) Contrast of the image as a function of the laser power for electrical and optical signals. The contrast of the electrical image (green data) reaches 98% between 2 and 4 mW, whereas optical contrast (orange data) constantly decreases from a value of 62%. (C) SNR of the image as a function of laser power. Electrical SNR (green data) is limited by cross-talk noise and increases with the laser power. Optical SNR (orange data) is limited by photon shot noise and slowly decreases owing to growing background.

compatible with technology used in the electronics industry. This method offers advantages compared to conventional optical techniques, such as the high charge-carrier detection rate, which does not saturate with the applied optical power, and the high signal contrast even in circumstances where confocal detection cannot provide it. We anticipate that electrical read-out will boost fabrication of compact magnetometers and recently established lab-on-a-chip devices (19) for biology and medicine. Because this technique is capable of single charge-carrier generation on demand, it can be applied in quantum metrology as a standard for the electrical unit ampere (20). Moreover, this technique can be potentially transferred to new defects in diamond (21–23) as well as to other wide-bandgap semiconductors (24), where defects can be ionized.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/728/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S5
References (25–29)

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NANOMATERIALS

Chemically reversible isomerization of inorganic clusters

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Structural transformations in molecules and solids have generally been studied in isolation, whereas intermediate systems have eluded characterization. We show that a pair of cadmium sulfide (CdS) cluster isomers provides an advantageous experimental platform to study isomerization in well-defined, atomically precise systems. The clusters coherently interconvert over an ~1-electron volt energy barrier with a 140-milli-electron volt shift in their excitonic energy gaps. There is a diffusionless, displacive reconfiguration of the inorganic core (solid-solid transformation) with first order (isomerization-like) transformation kinetics. Driven by a distortion of the ligand-binding motifs, the presence of hydroxyl species changes the surface energy via physisorption, which determines “phase” stability in this system. This reaction possesses essential characteristics of both solid-solid transformations and molecular isomerizations and bridges these disparate length scales.

Phase transitions in solids and molecular isomerizations occupy different extremes for structural rearrangements of a set of atoms proceeding along mechanistic pathways. Phase transformations are initiated by nucleation events (1) that are difficult to define and then propagate discontinuously from lattice defects with activated regions smaller than the crystalline grains (incoherent transformation) (2). Small-molecule isomerization is a discrete process, in which the activation volume of the transition state is comparable to the size of the molecule (coherent transformation). Studies of isomerization and solid-solid transformations have thus far proceeded largely independently. Efforts to identify a system bridging these transformations have been made by examining the transformation of domains of reduced size, such as nanocrystals. Transformations of nanocrystals (100 to 10,000 atoms) do not mirror molecular isomerization, in that bulk-like phase transition behavior extends to the nanometer-length scale, even down to ~2 nm (2). Here we investigate the structural transformations in semiconductor cluster molecules at the boundary between molecular isomerizations and solid-solid phase transitions in nanocrystals (Fig. 1) by studying magic-size clusters (MSCs) (~10 to 100 atoms), as prototypical systems. Studies of these clusters (diameter < 2 nm) with distinct chemical formulas revealed that the cluster structures were strongly influenced by the surface termination (2–5).

Previous work has observed that certain types, or families, of MSCs can be converted into other MSCs (5–8). Thus far, however, experiments claiming to have observed structural reorganization have been primarily conducted in the solution phase. Clusters in solution are free to interact with each other and with unbound surfactants, monomers, or by-products, and these interactions promote mass transport and etching processes. For example, reports on InP clusters show irreversible structural changes, aggregation, and etching in the presence of high concentrations of amines (5). Such cases indicate a loss in the products’ compositional integrity and thus that the transformation is not an isomerization. Structural transformations have been proposed for the same InP clusters at lower amine concentrations (5) and in CdS clusters after changes in temperature (6). In the former case, the assignment to a structural transformation was made by indirect methods (9) based on changes in ³¹P nuclear magnetic resonance shifts. This measurement permitted identification of only ~20% of the atoms

in the cluster, none of which were directly associated with the surface ligands, and the experiment did not rule out the possibility of etching. Substantial changes in the ³¹P spectrum were observed in different solvents, bringing into question the dynamical stability of InP clusters in solution and, by extension, their status as isolated molecules undergoing discrete transformations. For the CdS clusters (6), the kinetics of incomplete transformations between cluster types indicated a very high activation energy (~3 eV), which is likely too large to account for merely structural reorganization energies and points instead to interparticle interactions. A primary complication of these solution-phase studies has been the lack of direct characterization of atomic structure, such as x-ray total scattering, in the native environment of transformation (5, 6) that can be used to identify the existence and extent of a structural transformation (9).

We demonstrate that a class of MSCs whose local structures can be modeled with a composition of Cd₃₇S₂₀ undergoes reversible isomerization between two discrete and stable states via a chemically induced, diffusionless transformation. We preserved the composition by isolating our clusters in solid films and determined the cluster structures (fit residuals < 0.2) through analysis of their x-ray pair distribution functions (PDFs). Switching between the isomers was triggered by the absorption-desorption of water or alcohol (hydroxyl groups) with an activation barrier of ~1 eV in both directions. This chemically induced, reversible transformation has characteristics of both molecular isomerization and bulk solid-solid transformations. These clusters are an attractive starting point for merging the long- and short-length scale descriptions of such transformations as mediated by the external surface energy.

We synthesized high-purity clusters (i.e., single product), characterized by a narrow excitonic absorption peak at 324 nm with negligible longer-wavelength absorption, via our high-concentration method (10). These clusters are stabilized by their mesophase (11) and immobilized in a thin solid film (Fig. 2A). We refer to this cluster type as α-Cd₃₇S₂₀. After exposure of

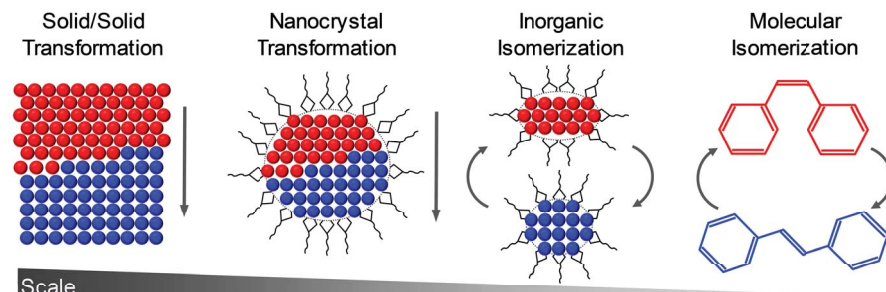


Fig. 1. Inorganic isomerization. Isomerization is well established in small organic molecules (e.g., the cis-to-trans transformation of azobenzene), whereas bulk inorganic solids exhibit phase transformations. Although small in size, nanocrystals follow bulk-like behavior in their solid-solid transformations. At even smaller length scales, inorganic clusters isomerize with molecular- and inorganic solid-like characteristics. Red and blue indicate two different structures.

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α -Cd₃₇S₂₀ films to methanol vapor, the exciton (first absorption) peak diminished, and a second narrow absorption peak emerged at 313 nm, indicating formation of the new species, β -Cd₃₇S₂₀, with an energy gap that is larger by 140 meV. Hereafter, only transformations with methanol are discussed in detail, but any hydroxyl-bearing species (alcohol or water) can initiate conversion of α to β .

The β -Cd₃₇S₂₀ could be transformed back to α -Cd₃₇S₂₀ (reversion) by purging the methanol and heating the MSC film (>60°C), and the reversion rate increased with temperature. We demonstrated a high degree of reversibility with four complete conversion-reversion cycles (inset of Fig. 2A) in the MSC isomerization without creation of other MSC families or nanocrystals (fig. S1D). This behavior in MSCs is reminiscent of reversible isomerization reactions, which are well known in small molecules (12). We observed that differences in the dielectric environment only weakly alter the absorption maximum wave-

length and that, indeed, the presence of hydroxyl-bearing species exclusively determines the favored isomer under the temperatures applied here. α -Cd₃₇S₂₀ may be stabilized at lower temperatures by maintaining an anhydrous environment, and β -Cd₃₇S₂₀ may be stabilized at higher temperatures (e.g., up to the boiling point of methanol) by maintaining the saturation of hydroxyl-bearing species. The stabilization of different MSC forms within a mesophase may have interesting consequences for nanoparticle formation once growth (e.g., by oriented attachment) is initiated, as mentioned in recent reports (13).

Both MSC isomers had low photoluminescence (PL) quantum yields (<2.5%), which indicates that nonradiative decay processes dominated at room temperature. Substantial emission from the clusters' electronic transitions was present (Fig. 2B). The PL decay transients could be fit by double exponentials (inset of Fig. 2B); the lifetimes of the slower decay rates are 5.8 and

5.2 ns for α -Cd₃₇S₂₀ and β -Cd₃₇S₂₀, respectively. The corresponding nonradiative and radiative rate constants for α -Cd₃₇S₂₀ are therefore 4.3×10^6 and 1.7×10^8 s⁻¹, respectively; for β -Cd₃₇S₂₀, they are 3.3×10^6 and 1.9×10^8 s⁻¹, respectively (see the supplementary materials for calculations).

We analyzed the structure of the isomers by x-ray diffraction (XRD), noting that the XRD shoulder at $2\theta \sim 37^\circ$, where 2θ is the angle between the incident and the diffracted x-ray beam, in the α -Cd₃₇S₂₀ is absent in the β -Cd₃₇S₂₀ (Fig. 2C). Although the peaks are broad, we interpret the α and β isomers as generally having “wurtzite-like” and “zinc blende-like” phases, respectively. We resolved the detailed atomic structure of the cluster isomers by fitting the PDF derived from the total scattering function [$G(r)$] using a Monte Carlo algorithm (see supplementary materials and methods). The best-fit structures of α and β (residuals of ~ 0.18 ; fig. S2, B and C) were comparable to InP clusters (14) (formula unit: In₃₇P₂₀), but with the In and P atoms replaced

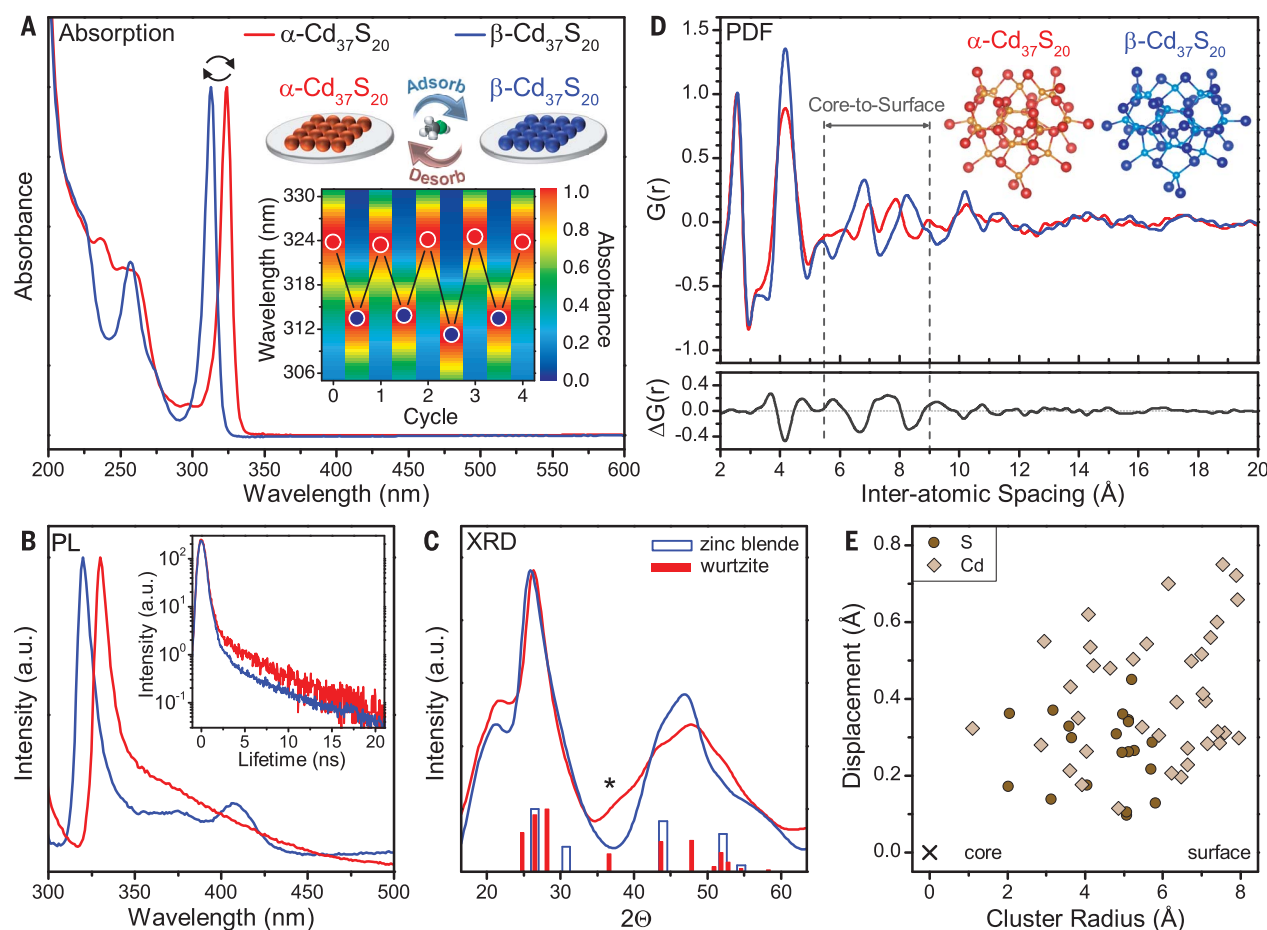


Fig. 2. Electronic and structure analysis. (A) Absorption spectra of pristine cluster isomers α -Cd₃₇S₂₀ and β -Cd₃₇S₂₀, with excitonic peaks at 324 and 313 nm, respectively. The two isomers switch reversibly upon alcohol adsorption and desorption (inset schematic and contour plot). The slight deviation between cycles is associated with ambient temperature fluctuations. (B) PL and lifetime (inset) of the isomers. a.u., arbitrary units. (C) Synchrotron XRD patterns of α and β isomers referenced to a Cu-K α source [wavelength (λ) = 0.154 nm]. Peak positions for the wurtzite

(with defining feature at 37° , asterisk) and zinc-blende phases of CdS are from the Joint Committee on Powder Diffraction Standards card numbers 00-041-1049 and 00-010-0454, respectively. (D) PDFs of the α and β isomers. $\Delta G(r) = G_\alpha(r) - G_\beta(r)$ is the difference in the PDF between the two isomers and is largest for core-to-surface atom pair distances. Inset are the fitted structures of the α and β isomers with residuals of ~ 0.18 . (E) Radial displacement of atoms between the α and β isomer structures with respect to distance from the cluster geometric center (X).

by Cd and S, respectively. PDF analysis is an effective tool for atomic modeling that resolves fine features and subtle differences between data. Although powerful for low-symmetry and disordered systems, extracting atomic positions from PDF analysis and modeling hinges on the accuracy of the initial inputs (15, 16). Repeated fitting showed that α - and β -Cd₃₇S₂₀ structures occupied distinct energy minima whose separation greatly exceeded possible overlap from thermal displacements, so that the clusters' structures are unambiguously different (fig. S2D). Simulations including contributions from the organic ligands and the mesophase assembly determined that the organic ligand shell does not substantially contribute to scattering above $Q = 1.5 \text{ \AA}^{-1}$, where scattering from the inorganic structure is dominant, so that fitting $G(r)$ beyond 2 Å even without organic contributions correctly resolves the positions of Cd and S (fig. S2, B and C). Our structures have a low symmetry (inset of Fig. 2D), unlike the highly symmetric tetrahedral coordination that has been reported for other CdS or CdSe MSCs (16, 17). We hypothesize that our clusters resemble the InP structure because our clusters are similarly passivated with only carboxylate ligands, whereas previously reported CdS or CdSe clusters are stabilized by amines, thiols, or a mixture of ligands. The representative structures of the clusters are molecular-

like but have scattering features similar to CdS crystal phases.

The difference between the α and β PDFs, $\Delta G(r)$, indicates changes in the atomic positions (Fig. 2D), for which larger magnitudes signify a greater shift between the structures. Although $\Delta G(r)$ revealed preservation of the CdS bond lengths [$\Delta G(r) \approx 0$ between 2.50 and 2.55 Å], there were appreciable differences in the bond angles [$\Delta G(r) \neq 0$ between 4 and 5 Å]. Analysis of our atomic structures indicated an overall broader distribution of bond angles in the α -Cd₃₇S₂₀ than the β -Cd₃₇S₂₀ (fig. S2, E and F). These changes in conformation (atomic orbital overlap) must be the origin of the change in the excitonic gap between clusters. The greatest difference between the PDFs of the isomers was within the range of 5.5 to 9 Å, a range that corresponds to atomic pairs composed of one "core" atom and one "near-surface" atom.

Beyond an interatomic spacing of 12 Å, the $G(r)$ has oscillations that propagate to larger spacings (>30 Å). These features correspond to preferred intercluster orientations (diffraction texturing), which are broadened by variations in the cluster-cluster orientations (18). Our previous investigation revealed that these clusters form long-range assemblies (11). Although texturing or preferred nanograin orientation can create challenges in structural analysis by x-rays, these

challenges are less substantial in PDF analysis (18–20). To assign a degree of transformation, we calculated the set of displacements required to transform one cluster into the other (Fig. 2E). The resulting relative displacements between isomers increases with radial distance from the cluster's geometric center. Despite the large magnitude of displacement (up to ~30% of the Cd–S bond length for surface atoms), the connectivity of α - and β -Cd₃₇S₂₀ does not change. Therefore, the cluster isomerization is primarily displacive, characteristic of a solid-solid transformation, rather than reconstructive.

The Fourier transform infrared (FTIR) spectra of α and β reveal that the isomerization stems from changes in the surface structure. We identify the carboxylate asymmetric stretches (ν_{as}) at 1528 and 1538 cm^{−1}, respectively (Fig. 3, A and B), and the carboxylate symmetric stretches (ν_s) at 1410 cm^{−1} for both isomers. The difference (Δ) between ν_{as} and ν_s gives the ligand-binding motif: $\Delta < 140 \text{ cm}^{-1}$ indicates a chelating bidentate configuration, and $\Delta > 140 \text{ cm}^{-1}$ indicates a bridging bidentate configuration (Fig. 3C) (21). The dominant ligand configuration in the α and β isomers is the chelating bidentate configuration ($\Delta_\alpha = 118 \text{ cm}^{-1}$; $\Delta_\beta = 128 \text{ cm}^{-1}$), but there is a strong shoulder in the ν_{as} (1580 cm^{−1}) in the α -Cd₃₇S₂₀ spectrum that points to the presence of some bridging ligands ($\Delta = 170 \text{ cm}^{-1}$) (22).

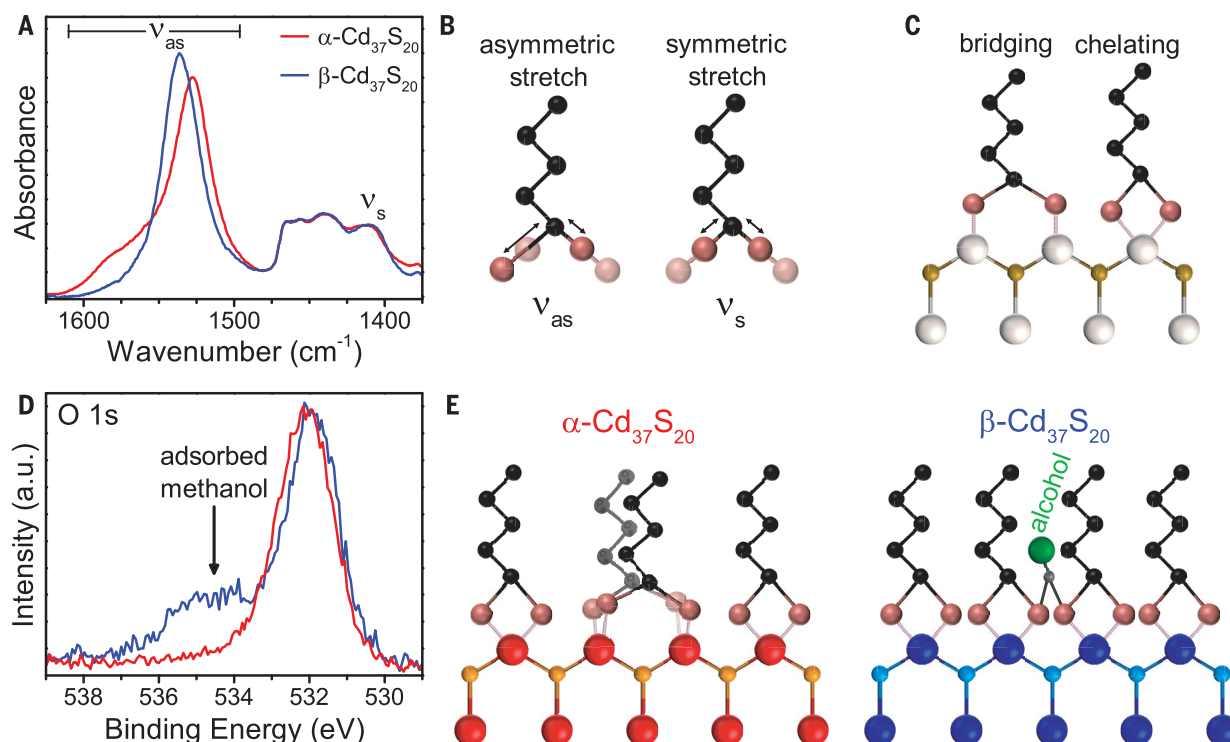


Fig. 3. Organic surface analysis. (A) FTIR spectra of the carboxyl asymmetric (ν_{as}) and symmetric (ν_s) stretches of the α -Cd₃₇S₂₀ and β -Cd₃₇S₂₀ isomers. (B) Schematic of the carboxylate stretch vibrations. (C) Observed bidentate carboxylate binding motifs. (D) O 1s XPS spectra in the α and β isomers. (E) Schematic of the ligand configuration on the isomer surface with chelating bidentate oleate

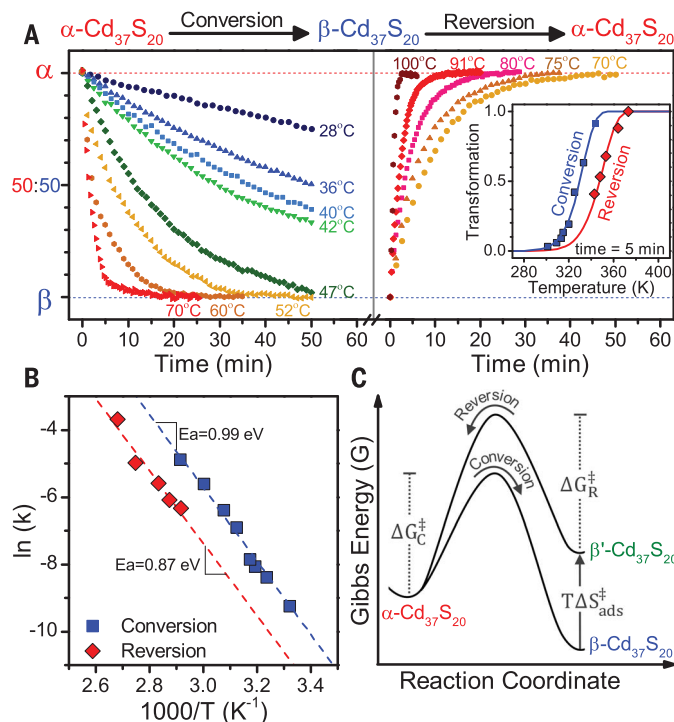
molecules. Methanol (green) hydrogen bonds with the oleate ligand to alter the chelating angle, which is larger in β -Cd₃₇S₂₀ relative to α -Cd₃₇S₂₀. Black, carbon; rose, oxygen; gray, cadmium; dark yellow, sulfur; red, α -Cd₃₇S₂₀ cadmium; orange, α -Cd₃₇S₂₀ sulfur; dark blue, β -Cd₃₇S₂₀ cadmium; light blue, β -Cd₃₇S₂₀ sulfur. Only one oleate is shown per cadmium atom for clarity.

Although this shoulder was absent in β - $\text{Cd}_{37}\text{S}_{20}$, the spectral area of the ν_{as} between the isomers is preserved, implying no change in the overall ligand number. From the correlation of bond angles from single-crystal diffraction data to FTIR spectra for various metal carboxylates (fig. S3B), we estimated the change in the two sets of bond angles from Δ (23, 24): The change in the chelating bond angle increased by $\sim 0.5^\circ$ upon conversion from α to β , and ligands changing from bridging to chelating configuration in the β - $\text{Cd}_{37}\text{S}_{20}$ decreased their bond angle by $\sim 2.0^\circ$. The FTIR results indicate that the cluster isomerization is strongly coupled to a change in the ligand-binding modes. We hypothesize that the modified ligand-binding arrangement on the cluster surface is the chemical trigger to the isomerization.

X-ray photoelectron spectroscopy (XPS) showed that the Cd 3d spectra for α and β were not notably different (fig. S4A and tables S1 and S2), suggesting little interaction of the Cd atoms with adsorbed methanol. However, the O 1s spectrum for α - $\text{Cd}_{37}\text{S}_{20}$ showed a peak at 531.9 eV, which shifts to 531.7 eV in the β - $\text{Cd}_{37}\text{S}_{20}$ spectrum. A second peak for β - $\text{Cd}_{37}\text{S}_{20}$ was present at 534.2 eV, which is attributed to physisorbed methanol (Fig. 3D). There was no evidence of dissociated methoxy species, which would have an O 1s peak at energies < 532 eV (25, 26).

In combination, FTIR and XPS analyses indicate that the presence of methanol shifts the configuration of ligands bound to the surface of the cluster. Changes in the carboxylate angle result in a reconfiguration of Cd and S atoms at the cluster surface, initiating the overall isomerization of the cluster (Fig. 3E). Control experiments using aprotic solvents with strong to weak dielectric constants (acetone to perfluorohexane, respectively) (table S3) did not induce a transformation (fig. S6A). The β - $\text{Cd}_{37}\text{S}_{20}$ is formed after the adsorption of methanol on the surface of the cluster, which arises via hydrogen bonding with the oleate ligand (Fig. 3D). Hydrogen bonding, and not changes in the dielectric environment, distorts the carboxylate bond angle and initiates the necessary surface reconfiguration that induces the cluster isomerization. Interestingly, such a hydroxyl-triggered phase change in the similarly structured $\text{In}_{37}\text{P}_{20}$ cluster (fig. S5) was not spectroscopically observed (14). Why $\text{In}_{37}\text{P}_{20}$ lacked another stable polymorph under conditions similar to those applied here is not obvious. We suggest that further investigations should identify, with atomic precision, the differences in ligand conformation, binding, and density, between $\text{In}_{37}\text{P}_{20}$ and $\text{Cd}_{37}\text{S}_{20}$.

The absorption peak of the β - $\text{Cd}_{37}\text{S}_{20}$ red shifts to 320 nm (fig. S7A) if methanol is not present (e.g., in vacuum), forming another species that we term β' - $\text{Cd}_{37}\text{S}_{20}$. Reexposure to methanol rapidly regenerated β - $\text{Cd}_{37}\text{S}_{20}$. The details of the β and β' spectra were otherwise nearly identical, indicating that the β -like structure is metastable at low temperatures and that hydroxyl is only required as an initiator. The β -to- β' transition shows that adsorbed methanol is not an essential contributor to the electronic structure. Likewise,



respectively, are the same. β - $\text{Cd}_{37}\text{S}_{20}$ transforms to β' - $\text{Cd}_{37}\text{S}_{20}$ upon removal of adsorbed alcohol with an entropic shift ($T\Delta S_{\text{ads}}^{\ddagger}$).

there are no substantial differences between the β and β' XRD and PDF patterns (fig. S7D), implying that the desorbed methanol affects the excitonic gap by way of dielectric effects.

Because the spectral overlap between the exciton of α - and β - $\text{Cd}_{37}\text{S}_{20}$ was small, we performed in situ time-resolved spectroscopy measurements at temperatures of 25° to 100°C to extract kinetic rate constants (Fig. 4A, fig. S8A, and table S4) through the evolution of the first absorption peak of α - $\text{Cd}_{37}\text{S}_{20}$. The isomerization followed first-order reaction kinetics and had a small transformation hysteresis (inset of Fig. 4A). For α -to- β conversion, we kept the methanol partial pressure saturated; when the methanol partial pressure fell, the transformation deviated from first order. In a dry or high-temperature environment, the reverse transformation was also first order. The Arrhenius prefactor, A (Fig. 4B and table S5), was $3.4 \times 10^{12} \text{ s}^{-1}$, which corresponds to a vibrational frequency of a transformation across the transition state ($k_B T \hbar^{-1} = 6.2 \times 10^{12} \text{ s}^{-1}$ at 300 K, where k_B is the Boltzmann constant, T is temperature, and $\hbar$ is the Planck constant) and agrees with measured prefactors for adsorption-desorption and solid-solid transformation processes (27, 28). We observed a smaller reversion prefactor ($9.3 \times 10^9 \text{ s}^{-1}$), on the order of those observed in some solid-solid transformations (29). Correspondence between the kinetic parameters from the optical experiments to those found from in situ diffraction confirmed the lack of structural intermediates (fig. S8, F to H), as did the isosbestic points in the optical absorption (fig. SIC).

The activation energy (E_a) values for the conversion and reversion processes were 0.99 ± 0.04

and $0.87 \pm 0.08 \text{ eV}$ (95.5 and 84.0 kJ mol^{-1}), respectively. In comparison, first-principles calculations have shown that the binding energy of carboxylic acids onto ($\text{Cd}_{33}\text{Se}_{33}$) is ~ 0.7 to 1.5 eV , with larger values for binding on higher-index facets (30). Compared to previously reported energies for a similarly described MSC that was unpurified, tested in dilute solutions, and only partially transformed, our activation energies are a factor of three smaller and align more closely with common structural transformation energies (i.e., solid-solid transformation and isomerization) (6). Our lower activation energy from more rigorous experiments better agrees with the low degree of local structural change during the conversion as inferred from direct characterization methods, such as pair distribution analysis.

We used the Eyring equation to derive the Gibbs free energy of the transition state, $\Delta G^{\ddagger}$ (table S6), and the apparent values for the enthalpy and entropy of the transition state ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, respectively) (Fig. 4C). The $\Delta H^{\ddagger}$ for the conversion and reversion processes are 0.96 ± 0.04 and $0.84 \pm 0.07 \text{ eV}$, respectively. The difference in $\Delta H^{\ddagger}$ between the processes may be related to the nonequilibrium desorption of physisorbed methanol in the reversion process. To investigate the possibilities of chemisorption and steric interactions, we performed the reversion process on β - $\text{Cd}_{37}\text{S}_{20}$ produced from alcohols with increasing alkyl chain length (fig. S8H) and found that $\Delta G^{\ddagger}$ was independent of the alcohol. We conclude that the $\Delta H^{\ddagger}$ is predominantly the free energy to relax the inorganic core after the change in the chemical potential at the ligand-core interface. Because we were unable to isolate the

Fig. 4. Transformation kinetics and thermodynamics.

(A) Kinetics of conversion and reversion processes. Both are first order: rate = e^{-kt} , where k is the rate constant and t is time. Inset is a hysteresis diagram for the transformed fraction at a reaction time of 5 min. (B) Arrhenius plot for the transformation kinetics with fits (dashed lines). $\ln(k)$, natural log of the rate constant. (C) Reaction coordinate diagram of the reversible transformation. The Gibbs free energies of the transition state for conversion and reversion, $\Delta G_C^{\ddagger}$ and $\Delta G_R^{\ddagger}$,

two isomers in coexistence with each other, the transformation must be kinetically controlled, and comparison to thermodynamic parameters, such as enthalpies of mixing [which are 1000-fold smaller (37)] cannot be made. The weak temperature dependence implies that the transformation is predominantly enthalpic, and the mean difference in $\Delta S^\ddagger$ of the transformation ($\Delta S^\ddagger_{\text{conversion}} - \Delta S^\ddagger_{\text{reversion}}$) of $+0.52 \text{ meV K}^{-1}$ is consistent with H-bonding entropies. As implied by Fig. 4C, the $\alpha\text{-Cd}_{37}\text{S}_{20}$ structure becomes thermodynamically unstable and converts into $\beta\text{-Cd}_{37}\text{S}_{20}$, owing to changes in the surface energy. Removal of the surface-energy perturbation by desorption of hydroxyl raises the free energy of $\beta\text{-Cd}_{37}\text{S}_{20}$, likewise rendering it thermally unstable and reverting to the α form.

A coherent transition between two clusters implies conservation of binding coordination, a single rate constant for the reaction, and simultaneous transformation of the entire cluster rather than growth from a nucleation site (2, 32, 33). On the basis of the transformation kinetics and the lack of any observable intermediates, the upper bound on the lifetime of an intermediate state must be on the order of 10^{-13} s (see supplementary materials for calculations), a time scale comparable to bond vibrations (33) and the lifetime of molecular transition states (34); additionally, to achieve the same rate of transformation for the MSC in an incoherent process, a phase boundary would need to move at a velocity comparable to the speed of sound of the bulk material (28, 35). The small atomic displacements shown from PDF analysis indicate a structural reconfiguration without a change in coordination number. Our experimental kinetics are thus consistent with a coherent atomic displacement occurring in a single step across the entire cluster, therefore bridging our understanding of molecular isomerization and solid-solid transformation.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S10
Tables S1 to S6
References (36–48)

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GEOPHYSICS

Inferring Earth's discontinuous chemical layering from the 660-kilometer boundary topography

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Topography, or depth variation, of certain interfaces in the solid Earth can provide important insights into the dynamics of our planet interior. Although the intermediate- and long-range topographic variation of the 660-kilometer boundary between Earth's upper and lower mantle is well studied, small-scale measurements are far more challenging. We found a surprising amount of topography at short length scale along the 660-kilometer boundary in certain regions using scattered $P'P'$ seismic waves. Our observations required chemical layering in regions with high short-scale roughness. By contrast, we did not see such small-scale topography along the 410-kilometer boundary in the upper mantle. Our findings support the concept of partially blocked or imperfect circulation between the upper and lower mantle.

The globally observed 660-km seismic discontinuity defines the top of the lower mantle and is commonly understood to involve the phase transition of the mineral ringwoodite to bridgmanite and ferropericlase. The detailed nature of this interface provides constraints on the chemical and dynamic properties of the whole mantle. Several lines of evidence support the boundary being due to the phase transition alone. If this is the case, the natural conclusion is that the whole mantle is convecting on geologic time scales (1, 2), despite the mineralogical differences between the upper and lower mantle. However, not all observations support this picture of the discontinuity. Other geochemical and mineralogical lines of evidence suggest a chemical interface, which requires some sort of dominantly layered or compartmentalized convection in order to maintain chemical differences between the upper and lower mantle (3–6). Seismic waves can be used to measure many features of the discontinuity related to the physical properties at the boundary, including sharpness, density, elasticity contrast, and topographic variations (7–11).

Topographic variation provides essential clues for understanding the nature of the 660-km discontinuity. Topography is the result of dynamic processes and the heterogeneous distribution of density. The free surface and the core mantle boundary of Earth feature topography from scales of a thousand kilometers to a few kilometers (12–14), leading to the expectation that the 660-km discontinuity might also be

rough at many scales. The scale of roughness on a boundary provides insight into the dynamic processes responsible for the topographic variations.

Our current picture of the topography of the 660-km discontinuity comes from precursors of

reflected body waves (such as PP , SS , and $P'P'$) (7, 8, 10) or converted phases such as $P660s$ (15, 16) and $S660P$ (17, 18). These methods reveal the large-scale (~1000 km) topography from thermal variations (Fig. 1A) of up to tens of kilometers (7, 10). Intermediate-scale (~100 km) topography (Fig. 1A) has been mapped with receiver functions (15, 16) or converted phases (17, 18) because they have smaller Fresnel zones. Most of the intermediate- and large-scale topography results are interpreted as lateral temperature variations (19, 20), but some studies revealed that the 660-km topography may be associated with more complex mechanisms than just the phase transition (21–23).

In order to determine the small-scale (~10 km) topographic variations of the 660-km and the 410-km seismic discontinuities, we used the scattering of short period waves. This strategy has been successful for the upper crust, where $P'SurfP'$ waves were generated by asymmetric (out of plane) back-scattering of small-scale free-surface topography and/or heterogeneities in the upper crust (24). We used the $P' \cdot d \cdot P'$ phase (25), in which a rough interface is at a depth d below the surface of Earth (Fig. 1, B and C), and the double amplifications of PKP near its caustic distance can substantially enhance weak signals.

We chose seismic waveforms at small angular epicentral distances (roughly, 0° to 40°), at

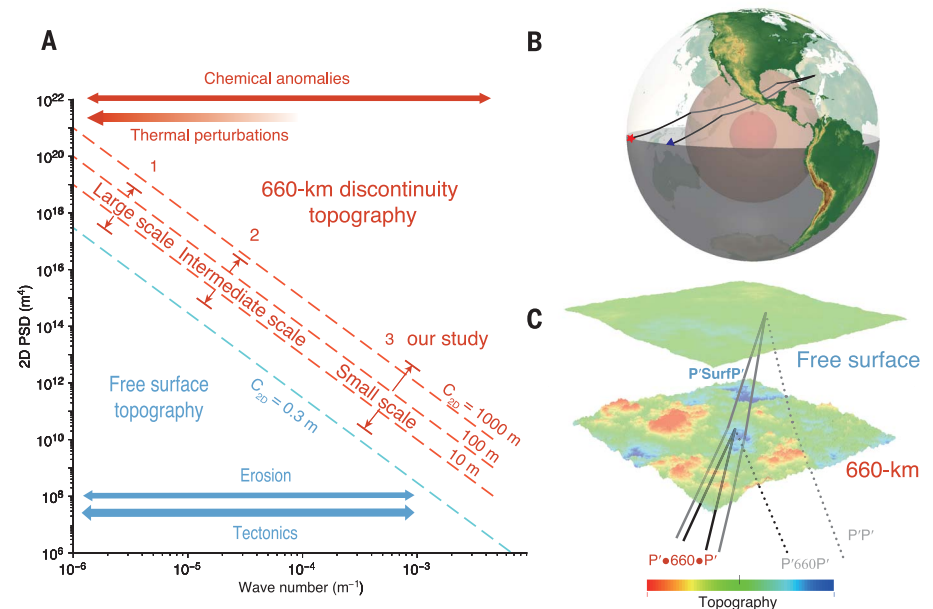


Fig. 1. PSD of the 660-km interface and ray path of $P' \cdot 660 \cdot P'$. (A) PSD of 2D free-surface topography (blue dashed line) and 660-km discontinuity topography (red dashed lines) as a function of wave number k (km^{-1}). The dashed blue line is $P = C_{2D}k^{-3}$ with $C_{2D} = 0.3$ m (33), and the red dashed lines represent $C_{2D} = 10, 100$, and 1000 m, respectively. The large, intermediate, and small lateral-scale ranges of the 660-km discontinuity topography are labeled as “1,” “2,” and “3” respectively. Intermediate-scale topography has not been thoroughly sampled because of the limited distribution of seismic stations. (B) Ray path of $P' \cdot 660 \cdot P'$. We set a fictitious source (red star) and receiver (blue triangle) on the equator. In contrast to most routinely reported seismic phases, which travel in a great circle plane (the equator in this figure), asymmetrical scattering permits out-of-plane scattering waves. (C) Cartoon ray paths of $P' \cdot 660 \cdot P'$ (black lines) and $P'SurfP'$ (gray lines) scattered from topography at the relevant interface. The black and gray dotted lines show ray paths of $P'P'$ and $P'660P'$, respectively, waves undergoing symmetrical reflections.

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which asymmetrical scattering waves $P'\bullet d\bullet P'$ are readily observable (24–26). Because the 660-km discontinuity has a much lower impedance contrast than the free surface (27), we carefully selected seismic stations with low noise levels and strong earthquakes (table S1) to enhance the chance of observing clear $P'\bullet 660\bullet P'$. We also used high-quality small-aperture seismic arrays such as IL (Eielson), YK (Yellow Knife), and AS (Alice Springs) to enhance signal-to-noise ratio (SNR) and perform beam-forming analysis. These three arrays have been used extensively to detect various types of weak signals because they have superb performance (25, 26, 28, 29).

The raw seismic data are usually noisy, so we used short period filtering (1.5 to 2.5 Hz, used throughout unless otherwise stated) to increase the SNR. After filtering, two strong signals $P'\bullet d\bullet P'$ are evident in the data (Fig. 2A). The strongest signals, arriving at ~2280 s, are mainly $P'\text{Surf}P'$, with some possible energy from $P3KP$ (24). The other clear signals arrive 150 to 160 s before the theoretical arrival time of $P'\text{Surf}P'$. This 150- to 160-s lead time is consistent with the time in-

terval between $P'\bullet 660\bullet P'$ and $P'\text{Surf}P'$, implying that the earlier distinct signals could be $P'\bullet 660\bullet P'$. If we take the smoothed envelope of this short-period seismogram (Fig. 2A, bottom trace), both the earlier signals and $P'\text{Surf}P'$ have spindle shapes, which suggests scattering as their origin (12, 30), although the SNR of $P'\bullet 660\bullet P'$ is not high.

In order to confirm the nature of the probable back-scattered seismic signals, we analyzed seismograms at the IL array from a different earthquake, beneath the Okhotsk Sea (Fig. 2B). Both $P'\text{Surf}P'$ and $P'\bullet 660\bullet P'$ are clear in the stacked result, and $P3KP$ arrives between them (Fig. 2B). The arrival time (~2130 s) and ensemble shape of $P'\bullet 660\bullet P'$ envelopes are similar to these at LPAZ (La Paz). Additionally, all the 19 smoothed envelope seismograms are very similar to each other, arguing for coherent and robust seismic phases rather than local noise. The stacked result yields a reliable observation, with a SNR greater than 2.

We collected more data of the three arrays (AS, IL, and YK) from 10 more earthquakes (table S1) and repeated the stacking process (Fig.

2C, stacked seismograms, and fig. S2, detailed data). We examined the National Earthquake Information Center (NEIC) earthquake catalog (31) and ruled out the possibility of contaminating signals from any aftershock in the time window of concern. With angular epicentral distances from podal distance (0°) to $\sim 40^\circ$, the arrivals of $P'\bullet 660\bullet P'$ are almost constant ~160 s before $P'\text{Surf}P'$ arrivals (Fig. 2C). A constant travel time, independent of angular epicentral distance, is the specific character of asymmetrical back-scattering (out of great-circle plane scattering) (24), in contrast to other commonly observed seismic phases traveling on a great-circle path (for example, Fig. 2C, $P3KP$). Thus, we confirmed that the signal is $P'\bullet 660\bullet P'$. However, we did not see the $P'\bullet 410\bullet P'$ signal from the 410-km boundary.

An advantage of array data is that directional information can be resolved for seismic signals in the form of wave slowness (28), which is valuable for identifying asymmetrical back-scattering (24, 25). We used the larger-aperture (~20 km) YK and AS arrays to investigate the slowness of $P'\bullet 660\bullet P'$. Smaller-aperture arrays (such as the

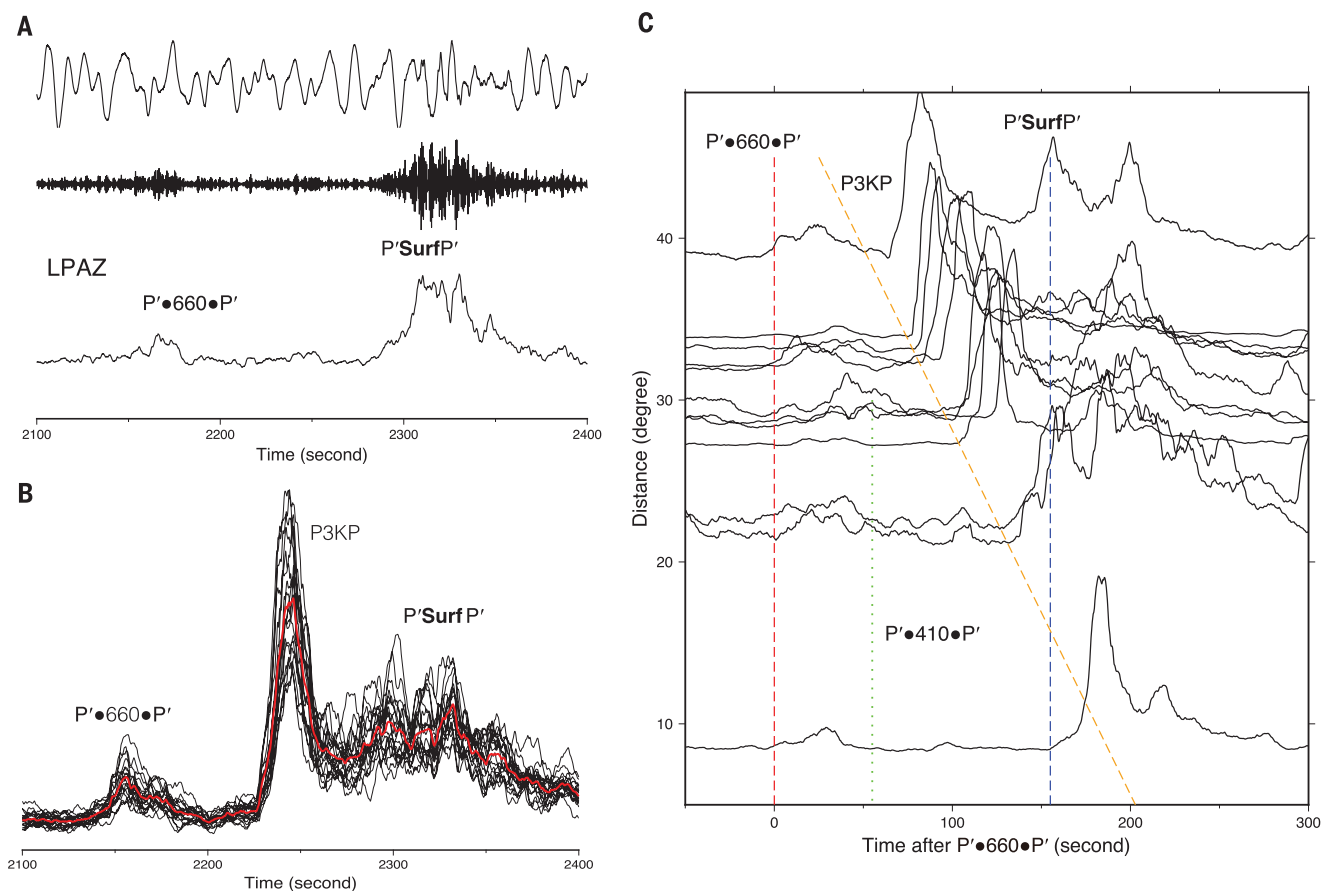


Fig. 2. $P'\bullet 660\bullet P'$ observations from individual stations and arrays. (A) $P'\bullet 660\bullet P'$ observed at station LPAZ for the 20 June 2003 M_w 7.0 South America earthquake. The top trace is broadband velocity waveform data. The middle and bottom traces are high-frequency (1.5 to 2.5 Hz) filtered data and its smoothed envelope, respectively. (B) Smoothed envelope observed at the IL array from the 24 November 2008 M_w 7.3 Okhotsk Sea earthquake. Black lines represent the smoothed-

envelope seismograms of the 19 constituent stations, and the red line is their stacked trace. (C) Stacked smoothed envelopes at AS, IL, and YK arrays from 11 events. Each trace represents stacked data of an array from one event, equivalent to the red line in (B). The three dashed lines are the predicted arrivals of $P'\bullet 660\bullet P'$ (red), $P3KP$ (orange), and $P'\text{Surf}P'$ (blue), respectively. The dotted green line shows the arrival of $P'\bullet 410\bullet P'$.

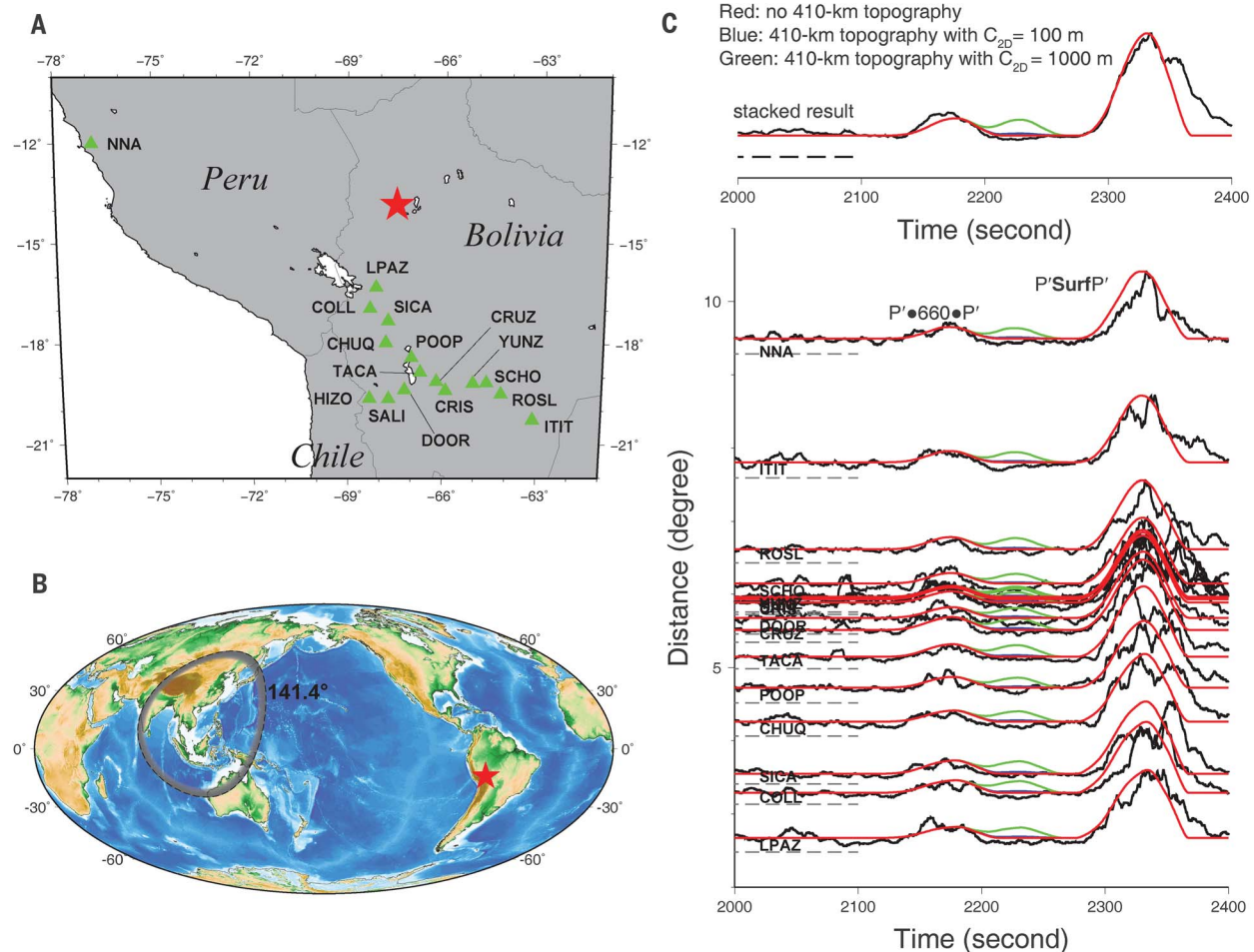


Fig. 3. Maps, synthetic and observed envelopes of $P'•660•P'$ at near podal distances from the M_w 8.2 Bolivia earthquake. (A) Map of the earthquake (red star) and seismic stations (green triangles) used in (C). (B) The sampled region of $P'•660•P'$ (gray area) for this earthquake (red star). The gray area starts from the PKP caustic distance of 141.4° on the 660-km discontinuity and gradually vanishes because of the decaying amplitude of PKP with increasing distance. (C) Observed and synthetic smoothed envelopes of high frequency (1.5 to 2.5 Hz) $P'•660•P'$, $P'•410•P'$, and $P'SurfP'$. Smoothed envelopes of velocity seismograms of observations (corresponding to black lines)

and synthetics (colored lines) are plotted with normalized amplitude. The black dashed lines indicate the zero baselines of the seismograms. C_{2D} for the 660-km discontinuity topography models is 100 m for all the synthetics. $P'SurfP'$ is generated by volumetric heterogeneities with exponential autocorrelation function (autocorrelation length $L = 13$ km and perturbations root mean square = 5%) in the top 10 km crust (24) and free surface topography (a power law PSD with $C_{2D} = 0.3$ m). Taking $P'SurfP'$ as reference, synthesized $P'•660•P'$ with $C_{2D} = 100$ m seem to match the observations well. $P'•410•P'$ is invisible or very weak in observations.

10-km IL array) might have insufficient resolution. Signals arrive with azimuths of about $\pm 120^\circ$ off the great circle paths and slowness > 1.9 s/degree (fig. S3). These results are consistent with the slowness of $P'•660•P'$ at angular epicentral distance around 30° (24, 25) because of the asymmetrical back-scattering of PKP waves.

This type of asymmetric back-scattering can be generated by either volumetric heterogeneities near the 660-km boundary or the topography of the 660-km discontinuity (fig. S4). The arrival times of observed $P'•660•P'$ restrict the heterogeneities to a thin layer near the 660-km discontinuity. Either deeper or shallower scatters would cause substantial advance or delay of its travel time, contrary to our observations (fig. S5). Previous studies (32) did not observe the strong precursors or coda waves of PP' , which

would imply volumetric heterogeneities, so rough topography seems to be the more likely candidate.

The topographic variations of the 660-km discontinuity are constrained from the amplitudes of $P'•660•P'$. We used the power law $P = C_{2D}k^{-3}$, where C_{2D} indicates how rough the interface is for the two-dimensional (2D) power spectral density (PSD) of the 660-km interface and ray theory to model asymmetrical scattering synthetics (33). In order to reduce the uncertainties, we used $P'SurfP'$ as a reference phase to investigate $P'•660•P'$ because their ray paths are very close to each other.

As the second largest deep-focus event ever recorded (34), the 9 June 1994 moment magnitude (M_w) 8.2 Bolivia deep earthquake provides an opportunity to estimate the topography of the 660-km discontinuity by modeling the ratios of amplitudes of $P'•660•P'$ to $P'SurfP'$ (Fig. 3).

Sixteen broadband stations in Bolivia and Peru recorded strong $P'•660•P'$ and much stronger $P'SurfP'$, with angular epicentral distances from 2° to 10° (Fig. 3C, black lines). To obtain the theoretical ratio of $P'•660•P'$ to $P'SurfP'$, the synthesized envelope functions of $P'•660•P'$ (fig. S4A), $P'•410•P'$, and $P'SurfP'$ were formed by using the Preliminary Reference Earth Model (PREM) (27). The smoothed and squared envelope of the first 50 s direct P wave from the teleseismic station SJG (San Juan) was taken as an empirical source time function (fig. S6) and convolved with the squared synthetic envelope functions. The energy levels of background noise were estimated from the data by using the average of squared envelope in the time window 2000 to 2100 s (Fig. 3C, black dashed line) and then accounted for in the synthetic envelopes.

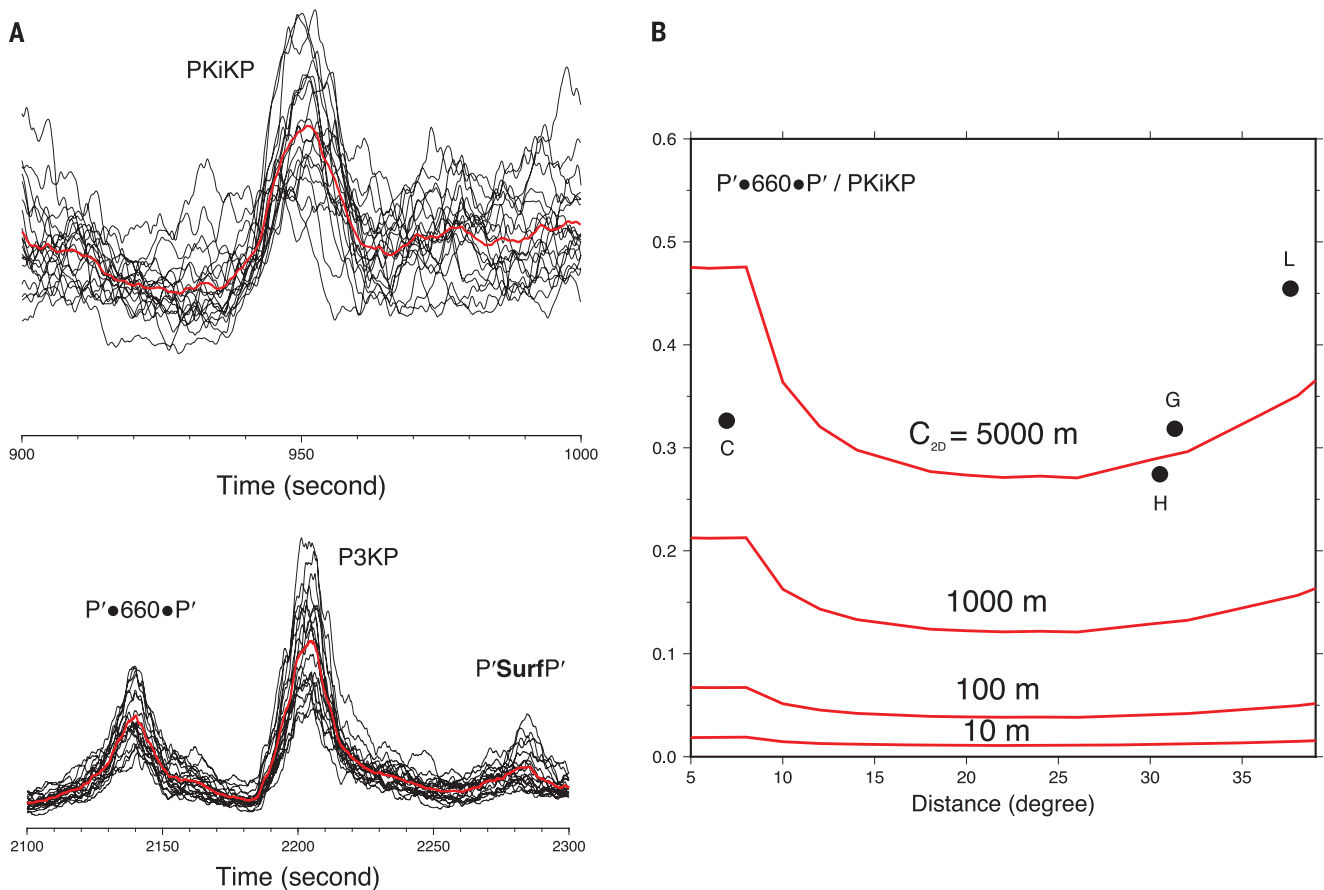


Fig. 4. $P'•660•P'$, PKiKP, and their amplitude ratios. (A) Smoothed envelopes of velocity seismograms of high-frequency (3 to 4 Hz) PKiKP (top) and $P'•660•P'$ (bottom) recorded on the IL array from the 14 August 2012 Sea of Okhotsk earthquake. The red traces are the stacked results. (B) $P'•660•P'/PKiKP$ amplitude ratios for four events. The envelope functions of $P'•660•P'$ (fig. S4) and PKiKP

were calculated by using ray theory. The red lines represent predicted $P'•660•P'/PKiKP$ amplitude ratios for four topography models with different coefficients C_{2D} (10, 100, 1000, and 5000 m). The measured $P'•660•P'/PKiKP$ amplitude ratios (circles) are higher than the red line with $C_{2D} = 1000$ m and well distributed around the line of $C_{2D} = 5000$ m.

$P'•660•P'$ synthetics (Fig. 3C, red lines) with $C_{2D} = 100$ m matched the data well in terms of both the onset and shapes, and we used this value as a reasonable estimation of the 660-km discontinuity topography for this sampled region. We attempted only to estimate the order of magnitude of C_{2D} rather than a precise measurement (fig. S7) because substantial uncertainties are involved owing to poorly constrained aspects of Earth's structure (33, 35). This order of magnitude of 100 m is much larger than the global average C_{2D} of the free surface ($C_{2D} = 0.3$ m) (Fig. 1A). We also computed $P'•660•P'$ from alternative models of volumetric heterogeneities around the 660-km boundary, and this heterogeneous layer, if present, must be thin (<250 km) (fig. S8). The durations of $P'SurfP'$ in the observations were longer than in the synthetics. This might be due to the presence of $P3KP$ arrivals in observations, which we did not account for in the synthetics.

The 410-km global discontinuity is associated with the olivine-to-wadsleyite phase transition. Similar to $P'•660•P'$, we would expect to see the asymmetrical scattering wave $P'•410•P'$ if

the 410-km is sharp and rough at small lateral scales. However, we do not discern $P'•410•P'$ on individual seismograms. In order to enhance its observability, we stacked all the smoothed envelopes and were still not able to identify $P'•410•P'$. This indicates weaker 410-km discontinuity small-scale topography than that of the 660-km discontinuity. One interpretation of this observation is very weak small-scale topography on a sharp 410-km discontinuity (Fig. 3C). This interpretation implies that the 410-km discontinuity is a pure phase-transition boundary, which only has topographic variations at large and intermediate scales mostly caused by smoothly changing thermal structures. An alternative interpretation is that a broad transition width of the 410-km discontinuity (22, 36, 37) could substantially reduce the reflection coefficient of short-period seismic waves and decrease the $P'•410•P'$ amplitude as the transition width approaches the wavelength of seismic waves. However, because other reports support a sharp rather than broad 410-km discontinuity (8, 38), the 410-km boundary would seem to be predominantly due to the phase transition.

$P'SurfP'$ travels twice as far in the upper mantle and crust as $P'•660•P'$, so uncertainty in the attenuation model of the upper mantle might bias our topography estimation. Furthermore, our synthesized $P'SurfP'$ also had large uncertainty because of poorly quantified small-scale heterogeneities in the crust (24). Upper-mantle and crustal structures affect our estimation of small-scale 660-km discontinuity topography. Thus, we used another phase—PKiKP—as a reference phase to estimate the topography of the 660-km discontinuity. PKiKP is often observed at high frequency, and its ray path is similar to that of $P'•660•P'$. Although $P'•660•P'$ travels four times through the lower mantle whereas PKiKP does so only twice, the lower mantle has much lower attenuation than the upper mantle (27, 39). By using PKiKP as a reference and PREM to describe the attenuation of both $P'•660•P'$ and PKiKP, we estimated 660-km discontinuity topography by comparing envelope synthetics to observations. After applying a high-frequency (3 to 4 Hz) filter, PKiKP is clear, and the SNR of $P'•660•P'$ is larger than 4 on the IL array for the

Sea of Okhotsk earthquake (Fig. 4A and table S1). Unlike the Bolivia earthquake (Fig. 3C), $P^*_{\text{Surf}}P'$ is even weaker than $P^*660\text{-}P'$, probably because of attenuation in the upper mantle. We compared the measured and synthetic ratios of $P^*660\text{-}P'$ to $PKiKP$ (Fig. 4B) and estimated a C_{2D} larger than 5000 m, which is several orders of magnitude larger than the globally averaged topography of the free surface. Factors such as uncertainty in the ICB reflection coefficient or topography may cause amplitude fluctuations of $PKiKP$ (40), thus biasing the estimation of 660-km topographic variation. However, the $P^*660\text{-}P'/PKiKP$ ratios measured at the IL array from other three events (Fig. 4B) show similar results of $C_{2D} > 1000$ m. This C_{2D} is much higher than the $C_{2D} = 100$ m estimated from the Bolivia earthquake (Fig. 3C). This substantial difference could be due to large uncertainty in the estimations of C_{2D} and/or geographical difference in the small-scale 660-km topography (fig. S9). Nonetheless, we observed strong topography of the 660-km discontinuity in both cases.

The $P^*660\text{-}P'$ in this study samples not only currently occurring deep subduction zones (such as Fig. 3B, Japan) but also regions without any known major subduction (figs. S10 and S11). In the former setting, a slab stagnant at the 660-km boundary (6) could accumulate a large amount of chemical heterogeneities, which cause 660-km small-scale topography and volumetric heterogeneities. In the latter case, accumulated oceanic crusts from ancient slabs could be buoyant above the 660-km boundary and form a garnetite layer filled with chemical heterogeneities (41).

Small-scale topography of the 660-km boundary, or a less likely thin layer of volumetric heterogeneities, would best be explained with a chemical origin. A gravitationally stable garnetite layer above the 660-km interface, due to oceanic crust accumulation from currently subducting and/or ancient slabs, is possible. This scenario was argued by Ringwood (42) and further discussed by Irifune and Tsuchiya (41). Some regions lack small-scale topography of the 660-km interface, implying a globally discontinuous chemical layer. Our observations support simulations that de-

scribe subducting slabs as transient features of the transition zone, which eventually penetrate into the lower mantle. They also support a picture of partially blocked upper- to lower-mantle circulation (43), which effectively alternates between layered and whole-mantle convection. This type of model is more complicated than either the layered or whole-mantle end-member case, but adding a time-dependent degree of material exchange over Earth's history may help unify geochemical, geodynamical, seismological, and petrological observations of the mantle.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/736/suppl/DC1
Materials and Methods
Figs. S1 to S11
Table S1
References (44–64)

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STRUCTURAL BIOLOGY

How a circularized tmRNA moves through the ribosome

Christopher D. Rae, Yuliya Gordiyenko, V. Ramakrishnan*

During trans-translation, transfer-messenger RNA (tmRNA) and small protein B (SmpB) together rescue ribosomes stalled on a truncated mRNA and tag the nascent polypeptide for degradation. We used cryo-electron microscopy to determine the structures of three key states of the tmRNA-SmpB-ribosome complex during trans translation at resolutions of 3.7 to 4.4 angstroms. The results show how tmRNA and SmpB act specifically on stalled ribosomes and how the circularized complex moves through the ribosome, enabling translation to switch from the old defective message to the reading frame on tmRNA.

Ribosomes that reach the 3' end of a mRNA without terminating at a stop codon stall in an unproductive state, forming “non-stop” translation complexes (1). In bacteria, these ribosomes are primarily rescued by trans-translation (2), without which the accumulation of nonstop translation complexes is inevitable and lethal (3–5).

Previous studies have described the roles of transfer-messenger RNA (tmRNA) and its binding partner, small protein B (SmpB), in trans-translation (6, 7). As its name implies, tmRNA contains both a tRNA-like domain and an mRNA-like domain (TLD and MLD, respectively) (fig. S1). The TLD resembles the acceptor arm of tRNA^{Ala}, which, together with SmpB, mimics a tRNA (7–9). tmRNA is charged with alanine (8) and binds elongation factor Tu (EF-Tu) for delivery to the

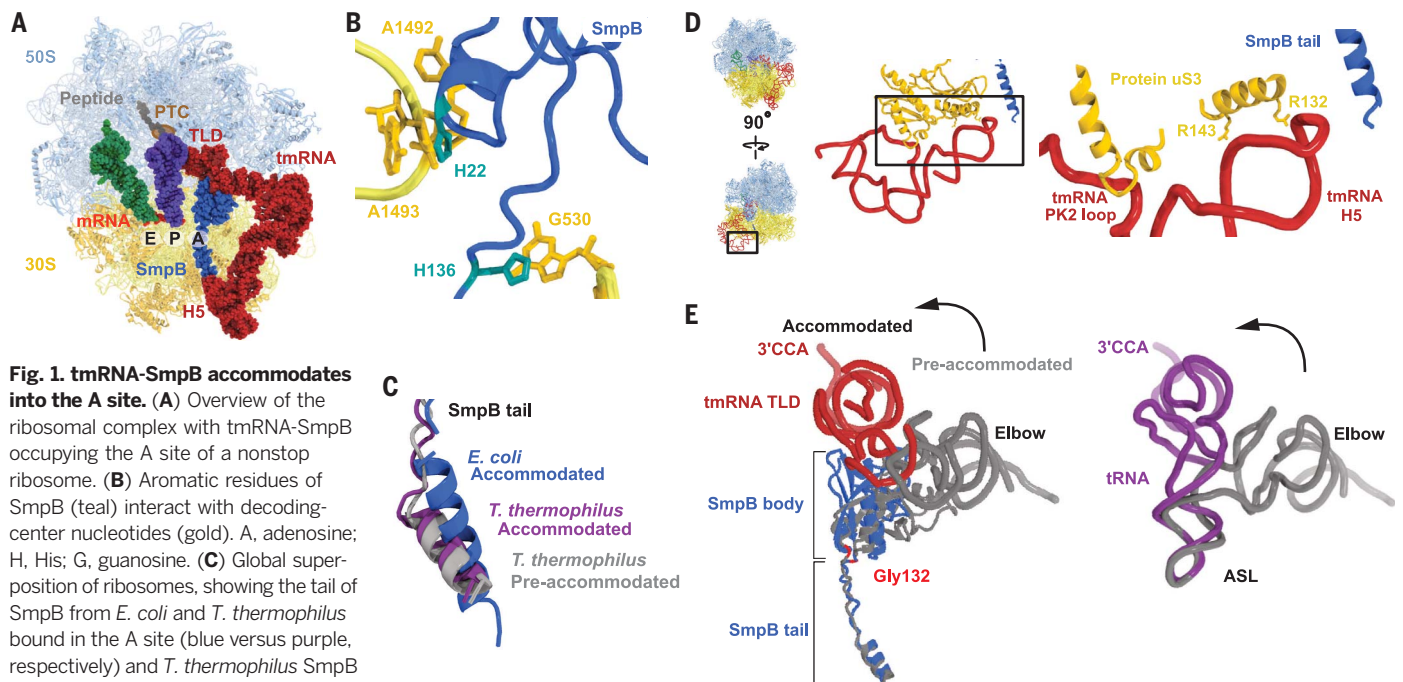
ribosome (10, 11). After accommodation into the A site, the nascent peptide is transferred to the alanine residue on tmRNA through a peptidyl transferase reaction. Elongation factor G (EF-G) then translocates tmRNA-SmpB through the ribosome in a manner similar to canonical translation (12). The ribosome swaps messages, abandoning the original mRNA and restarting translation on a reading frame in the MLD of tmRNA. Translation on tmRNA adds a polypeptide tag to the nascent chain and guarantees that the ribosome will terminate at a stop codon and be released. Despite previous low-resolution cryo-electron microscopy (cryo-EM) structures showing the overall position of tmRNA-SmpB in and between the A and P sites (12, 13), an atomic-level understanding of trans-translation is still lacking.

In this study, we used cryo-EM to determine the structures of three key trans-translation intermediates: tmRNA with the TLD-SmpB in the A site, P site, and a location past the E site of the 70S ribosome at 3.9-, 4.4-, and 3.7-Å resolution, respectively (fig. S2 and table S1). Nonstop ribosomes were affinity purified from an *Escherichia coli* in vitro translation system via 3xFLAG affinity tags on their nascent chains. Trans-translation was initiated by adding alanyl-tmRNA-SmpB-EF-Tu-GTP to the nonstop ribosomes to trap tmRNA-SmpB in the A site. The state after translocation, with tmRNA in the P site, was trapped as a result of including EF-G. The addition of Ala-tRNA^{Ala} with EF-G caused a second round of translocation to move tmRNA past the E site.

Full-length tmRNA-SmpB in the A site (Fig. 1A) binds a nonstop ribosome in two coordinated ways: (i) SmpB binds in the decoding center and mRNA channel, taking the place of a codon-anticodon interaction and downstream mRNA, and (ii) pseudoknot 2 (PK2) and helix 5 (H5) of tmRNA bind the solvent side of the ribosome and crowd the entrance of the mRNA channel. SmpB is “decoded” by the ribosome in a manner analogous but not identical to the usual codon-anticodon interaction in the A site. As in the pre-accommodated structure of the TLD-SmpB with EF-Tu trapped during delivery to the ribosome (14), His¹³⁶ of SmpB maintains a stacking interaction with G530. Additionally, the conserved aromatic residue, His²² of SmpB, stacks with

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A1493 (Fig. 1B). This observation is consistent with earlier biochemical probing experiments, which suggest that the binding of SmpB in the A site can protect decoding-center nucleotides (15). Unlike canonical decoding of tRNA, however, A1492 does not participate in decoding SmpB and remains only partially flipped out (14). Never-

theless, the small subunit is in a closed conformation resembling that of canonical decoding.

SmpB also occupies part of the mRNA channel (fig. S3A). Highly conserved, charged residues (fig. S3B) in the tail of SmpB interact with the mRNA channel to position SmpB in the A site. In the structure of *E. coli* tmRNA-SmpB bound

in the A site, the helix formed by the tail of SmpB is longer and shifted compared with that of the *Thermus thermophilus* pre-accommodated crystal structure (14) (Fig. 1C). The corresponding structure determined by using components entirely from *T. thermophilus* showed that the tail of SmpB remains in a nearly identical position

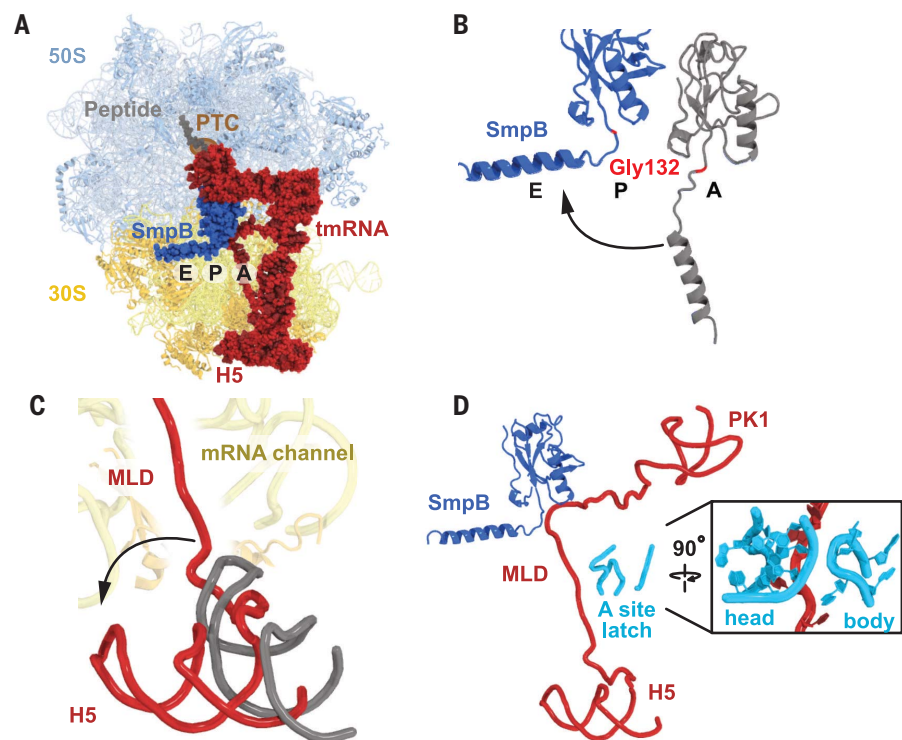


Fig. 2. tmRNA-SmpB is translocated into the P site of the ribosome.

(A) Overview of the ribosomal complex with tmRNA-SmpB bound in the P site and the MLD occupying the A site. (B) Comparison of SmpB occupying the A site (gray) versus the P site (blue). The tail of SmpB flips into the E site, anchoring tmRNA-SmpB in the P site. The conserved glycine residue at the junction between the body and the tail of SmpB is highlighted (red). (C) H5 of tmRNA changes position from the A site complex (gray) to the P site complex (red), allowing the MLD to pass through the space previously occupied by the tail of SmpB. (D) The MLD contacts the junction of the body and tail of SmpB to set the tag reading frame correctly in the A site. The MLD has passed through the A-site latch (inset) when tmRNA-SmpB occupies the P site.

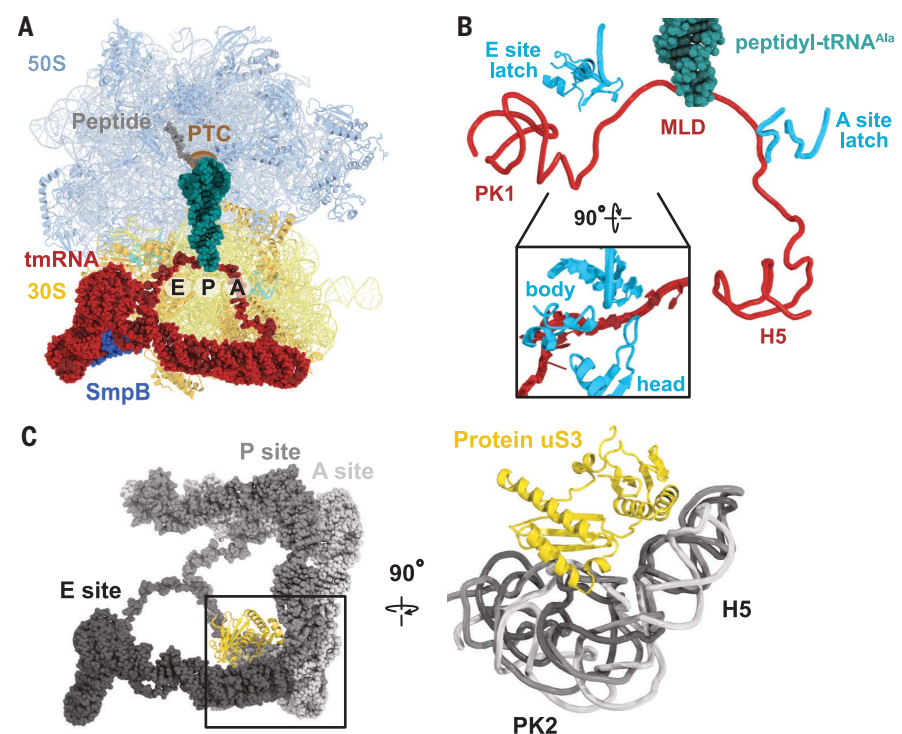


Fig. 3. tmRNA-SmpB is translocated from the P site past the E site.

(A) Overview of the ribosomal complex with tmRNA-SmpB bound on the solvent side of the E site after passing through the ribosome. Canonical tRNA (teal) bound to the resume codon occupies the P site. (B) Density for PK1, the MLD going through the mRNA channel, and H5 shows the MLD fully loaded into the mRNA channel. During the second translocation, the MLD passes through a second latch, this time in the E site (inset). (C) PK2 is anchored to protein uS3, acting as a flexible hinge point during the movement of tmRNA through the ribosome.

after accommodation (Fig. 1C), thus indicating that the shift is due to differences between species.

An interaction between a single-stranded loop of RNA from PK2 and ribosomal protein uS3 anchors tmRNA to the small subunit (Fig. 1D). This is consistent with the binding position of tmRNA in previous EM studies (12, 13). The loop of PK2 is sandwiched in a pocket of a helix-turn-helix motif of uS3. In this way, PK2 binding may explain why the tail of SmpB is dispensable for initial binding to the ribosome (16).

Additionally, H5 of tmRNA crowds the entrance of the mRNA channel in close proximity with the tail of SmpB (Fig. 1D). Arg¹³² and Arg¹⁴³ of protein uS3 make electrostatic interactions with the phosphate backbone of H5. Protein uS3 can similarly stabilize mRNA on the solvent side of the ribosome (17–19). Biochemical evidence shows that trans-translation activity is reduced on ribosomes that contain mRNAs with at least nine nucleotide extensions downstream of the

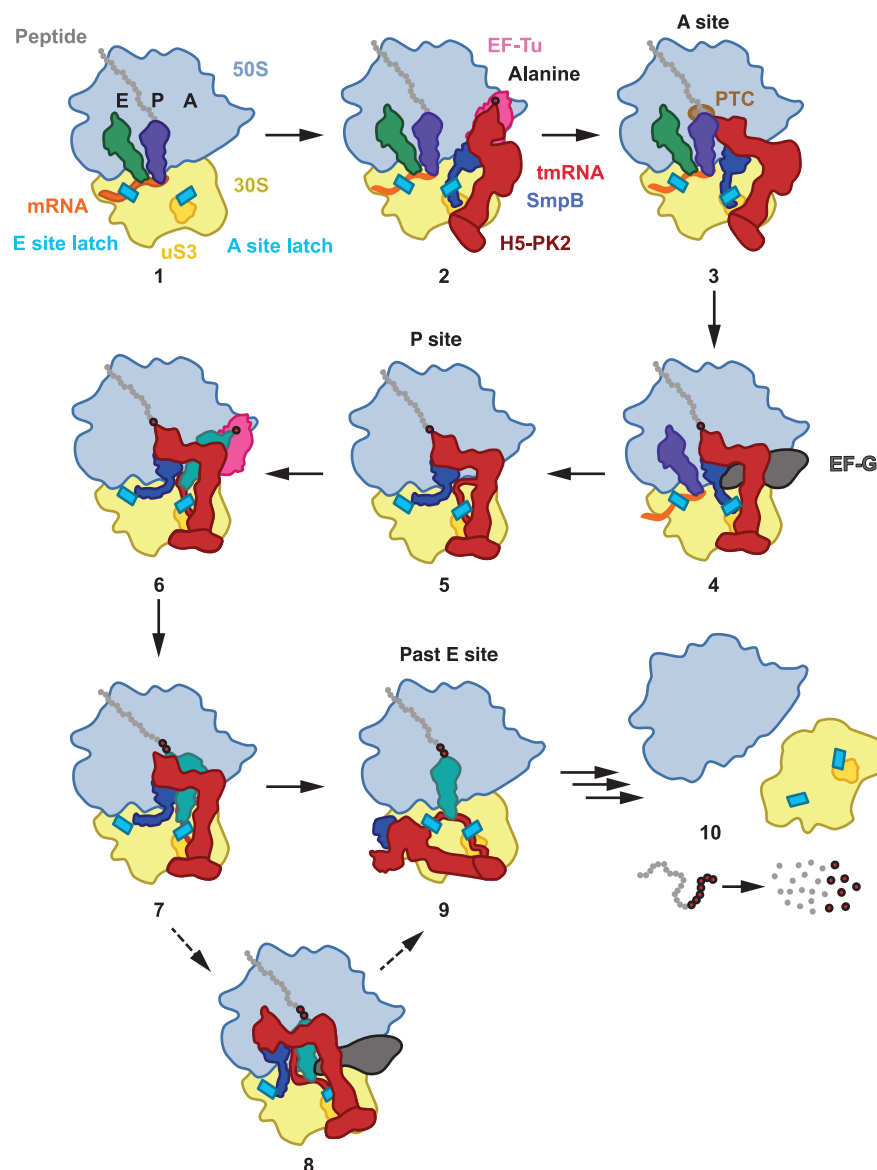
P site codon (20). Such an extension corresponds to the distance at which the mRNA would begin to clash with H5 at the entrance of the mRNA channel (fig. S3C). Binding of PK2 and H5 may therefore play a role in initial recognition of a nonstop translation complex.

During accommodation, tmRNA-SmpB undergoes a conformational change in two regions: (i) The highly conserved Gly¹³² in the tail of SmpB flexes to permit the body of SmpB to rotate into the A site, and (ii) the elbow region of tmRNA acts as a hinge around which the acceptor arm of the TLD swings into the peptidyl transferase center (PTC) (Fig. 1E and fig. S4). These movements are analogous to the distortions of the anticodon stem loop and elbow of canonical tRNA during accommodation (21).

After accommodation, the 3'CCA of tmRNA is positioned in the PTC, and the nascent peptide is transferred to the alanine on tmRNA during peptidyl transfer. Consistent with this notion, density

for the nascent peptide is seen in all three active *E. coli* structures (fig. S5). After peptidyl transfer, EF-G translocates tmRNA-SmpB into the P site, and the ribosome switches messages from mRNA to tmRNA (12) (Fig. 2A). The C-terminal tail of SmpB remains α -helical but flips to vacate the A site, binding the mRNA channel in the E site and anchoring tmRNA-SmpB in the P site (Fig. 2B). The highly conserved Gly¹³² facilitates flipping by again acting as a flexible joint between the body and tail of SmpB. In addition, H5 moves to unblock the entrance of the mRNA channel and permit the MLD to pass through (Fig. 2C). The movement of SmpB and H5 of tmRNA away from their original positions in the A site allows the MLD of tmRNA to occupy the mRNA channel. The MLD must pass through the A-site latch (also called the 30S latch) to load into the mRNA channel. The A-site latch is a physical barrier formed by the contact of the head (helix 34) and body (guanosine 530) of the 16S ribosomal RNA (rRNA) when the small

Fig. 4. Mechanism of trans-translation. (1) A 70S ribosome forms a nonstop translation complex when it stalls at the 3' end of a messenger RNA. (2) EF-Tu delivers Ala-tmRNA-SmpB to the ribosome where the C-terminal tail of SmpB forms an α helix in the downstream mRNA channel. When trapped in this state the complex is referred to as "pre-accommodated." (3) EF-Tu leaves and tmRNA-SmpB accommodates into the A site. The tail of SmpB remains in the same α -helical conformation as in the pre-accommodated state. Analogous to canonical tRNA, TLD-SmpB points the alanine on its 3'CCA into the PTC where it joins with the nascent peptide. PK2 interacts with protein uS3, binding tmRNA to the outside of the ribosome and coordinating the position of tmRNA as it moves through the ribosome. (4) EF-G translocates tmRNA-SmpB from the A site into the P site and expels the original mRNA and tRNA. (5) During translocation, the MLD passes through the A-site latch and into the space in the mRNA channel previously occupied by the tail of SmpB. The tail of SmpB flips to the opposite side of the mRNA channel, binding in the E site. (6) Ala-tRNA^{Ala} decodes the first codon of the reading frame of tmRNA (the resume codon), and (7) a peptidyl transferase reaction transfers the peptide from tmRNA to tRNA^{Ala}. (8) EF-G translocates the peptidyl-tRNA^{Ala} into the P site and consequently shifts tmRNA-SmpB (9) past the E site to the solvent side of the ribosome. During this second translocation event, the MLD is again loaded into the mRNA channel through a latch, this time at the E site. The MLD is fully loaded into the mRNA channel, and translation continues until terminating at a stop codon at the end of the reading frame. (10) The ribosome is released, and the peptide is targeted for degradation by proteases that recognize the polypeptide tag.



subunit is in a closed conformation. Ramrath *et al.* (12) suggest that passing through the A-site latch occurs via an extra large head movement that opens the latch during translocation.

Apart from loading the MLD into the mRNA channel, the reading frame within the MLD must also be positioned correctly for the ribosome to terminate at an in-frame stop codon. We can trace otherwise unassigned density leading from PK1 to SmpB across the A site and out of the mRNA channel, suggesting that the MLD interacts with the beginning of the tail of SmpB (Fig. 2D and fig. S5). This is consistent with biochemical evidence that indicates that the five nucleotides upstream of the “resume” codon, the first codon on tmRNA to be decoded, are critical for positioning the reading frame (22, 23).

Ala-tRNA^{Ala} decodes the resume codon on the MLD in the A site, and EF-G then translocates peptidyl-tRNA^{Ala} into the P site, consequently forcing tmRNA-SmpB toward the E site. Unexpectedly, tmRNA-SmpB does not mimic a tRNA bound in the E site. Instead, tmRNA-SmpB moves past the E site to the solvent side of the ribosome (Fig. 3A). Although it is formally possible that an intermediate E-site tmRNA-SmpB complex was skipped in the in vitro trans-translation system, superimposing tmRNA-SmpB from our structure onto a model of canonical tRNA in the E site induces clashes with the ribosome that make a stable E-site intermediate unlikely (fig. S6).

To complete loading into the mRNA channel, the MLD must pass through another latch, this time located at the E site. The E-site latch joins the head (protein uS7) and body (protein uS11 and guanosine 693 of 16S rRNA) of the small subunit. We see the MLD loaded into the mRNA channel after the second translocation step, analogous to the first (Fig. 3B). PK1 and H5 flank the single-stranded MLD, and density is seen running through the mRNA channel (fig. S5). The position of H5 is approximately the same as that of tmRNA-SmpB occupying the P site, thus continuing to provide room for the MLD to exit the channel.

During movement of tmRNA-SmpB between all three states, the single-stranded loop of RNA from PK2 remains bound to uS3, anchoring tmRNA to the solvent side of the small subunit (fig. S5). In this way, PK2 acts as a hinge about which tmRNA bends and pivots (Fig. 3C). The anchoring interactions of PK2 coordinate the different positions of H5 seen during trans-translation and limit the position of tmRNA on the solvent side of the ribosome after the second translocation event. PK2 is highly conserved (2), and its interactions with uS3 may therefore represent a general function of tmRNA.

As tmRNA-SmpB moves through the ribosome, SmpB first binds in the space subsequently occupied by the MLD after translocation. Thus, SmpB identifies legitimate nonstop ribosomes by verifying that the mRNA channel is empty and then vacates the space to make way for the MLD. Loading is necessarily mediated via a looping mechanism that passes through two latches, one during each translocation event (Fig. 4). Although the tmRNA we refer to here is a single, circularized molecule, this mechanism is likely applicable for two-piece tmRNA as well, because large secondary structures flank the MLD in many bacteria (24). This work shows how two translocation events move tmRNA-SmpB through the ribosome, resulting in complete loading of the MLD into the mRNA channel.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/740/suppl/DC1
Materials and Methods
Figs. S1 to S7
Table S1
References (25–42)
Movie S1

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STRUCTURAL BIOLOGY

Structural insight into nucleosome transcription by RNA polymerase II with elongation factors

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RNA polymerase II (RNAPII) transcribes chromosomal DNA that contains multiple nucleosomes. The nucleosome forms transcriptional barriers, and nucleosomal transcription requires several additional factors *in vivo*. We demonstrate that the transcription elongation factors Elf1 and Spt4/5 cooperatively lower the barriers and increase the RNAPII processivity in the nucleosome. The cryo-electron microscopy structures of the nucleosome-transcribing RNAPII elongation complexes (ECs) reveal that Elf1 and Spt4/5 reshape the EC downstream edge and intervene between RNAPII and the nucleosome. They facilitate RNAPII progression through superhelical location SHL(−1) by adjusting the nucleosome in favor of the forward progression. They suppress pausing at SHL(−5) by preventing the stable RNAPII-nucleosome interaction. Thus, the EC overcomes the nucleosomal barriers while providing a platform for various chromatin functions.

RNA polymerase II (RNAPII), a multisubunit protein factory (1), transcribes nucleosomal DNA to produce protein-coding mRNAs and many noncoding RNAs. A single nucleosome core particle includes a histone octamer, comprising two H2A-H2B dimers and an (H3-H4)₂ tetramer, wrapped with ~1.7 turns of DNA (2). Nucleosomes are inherent roadblocks of transcription, and RNAPII stalls at multiple locations within a nucleosome (3–5). We previously observed by means of cryo-electron microscopy (cryo-EM) that RNAPII stalls near the entry [superhelical locations SHL(−6) and SHL(−5)] and before the dyad [SHL(−2) and SHL(−1)] of the nucleosome, where the stalled RNAPII is maintained by tight histone-DNA contacts and direct RNAPII-nucleosome contacts (6). On the other hand, in cells the nucleosomal barriers are overcome by the dynamic, combinatorial actions of transcription elongation factors, histone modifications, histone chaperones, and nucleosome remodelers (7, 8).

Transcription elongation factors accompany RNAPII to facilitate efficient transcription through the nucleosome (9, 10). Elf1 and Spt4/5 are conserved basal elongation factors that are associated with transcribing RNAPII (11–13). Spt4/5, also known as DSIF in humans, is a heterodimer of Spt4 and Spt5 (14). Spt4/5 and its bacterial

homolog NusG stimulate transcription elongation by suppressing RNAP pause or arrest (15–17). Spt4/5 modulates the RNAPII processivity on nucleosomal DNA transcription (18). Elf1 (Elof1 in humans) is a small zinc finger protein (19) that has genetic interactions with other elongation factors—including Spt4, Spt5, Spt6, Spt16, and TFIIS—implying their functional relationships (20). A genome-wide profiling study suggested their roles in gene-body transcription (11). Both Elf1 and Spt4/5 also play a role in chromatin structure maintenance in actively transcribed genes (20, 21). However, it remains unclear how these factors allow RNAPII to overcome the nucleosomal barriers while maintaining the chromatin structure.

We examined the effects of Elf1 and Spt4/5 on nucleosomal DNA transcription. Transcription was performed by the yeast *Komagataella pastoris* RNAPII on the human nucleosome reconstituted with a modified Widom 601 DNA, as described previously (Fig. 1A and fig. S1) (6). RNAPII does not efficiently advance beyond the entry of the nucleosome [SHL(−5)] in the presence of TFIIS alone (Fig. 1B). By contrast, the addition of Spt4/5 allowed more efficient RNAPII progression to SHL(−1) or the DNA end (run-off). Elf1 exerted only a slight effect on the RNAPII progression on the nucleosome. However, the addition of both Elf1 and Spt4/5 exhibited a strong synergistic effect; together, they drastically reduced the pausing at SHL(−5) and dramatically increased the run-off product. Thus, Elf1 and Spt4/5 cooperatively lower the nucleosomal barriers, ensuring high elongation processivity on the nucleosome. This effect relies on TFIIS, which reactivates the stalled RNAPII (fig. S2) (22, 23).

To understand how Elf1 and Spt4/5 facilitate the nucleosome transcription, we analyzed the structure of the nucleosome-transcribing RNAPII elongation complex bound with these

factors (hereafter called the EC) (Fig. 1). The reconstituted nucleosome was transcribed by RNAPII in the presence of Elf1, Spt4/5, and TFIIS. The nucleosomal DNA contained a T-less region to enrich the EC at SHL(−1) in the presence of 3'-deoxyadenosine triphosphate (3'-dATP) (fig. S1A) (6). The EC-nucleosome complexes were prepared by means of the GraFix method (24) and subjected to cryo-EM analyses (Fig. 1C, figs. S3 to S9, and tables S1 and S2). Three-dimensional classifications revealed the EC-nucleosome complexes at SHL(−1) and SHL(−5), with ~60 and 20 base pairs (bp) DNA torn off from the histone surface, respectively (Fig. 1, D and E). The former complex is the one stalled at an intrinsic site(s) because it was also observed by using ATP (fig. S10). In these complexes, RNAPII-bound Elf1 and Spt4/5 were clearly observed, whereas TFIIS was missing, probably because it dissociated during the purification step. No discernible structures were obtained for ECs at SHL(−6) and SHL(−2).

In the EC-nucleosome structures, Elf1, the NGN domain of Spt5, and Spt4 form a domain array, which intervenes between RNAPII and the nucleosome (Fig. 1, D and E). Although no notable change was observed in the RNAPII structure, the relative RNAPII-nucleosome positions changed in the presence of the elongation factors as compared with those in their absence. The domain array occupies the double-stranded DNA-binding site used in the pre-initiation complex (25, 26), preventing the nucleosome interaction to the site (fig. S11). Elf1 intervenes between the Rpb1 clamp-head domain, the Rpb2 lobe domain, the downstream DNA, and the nucleosome, affecting the RNAPII-nucleosome interaction. The domain array also intervenes between the upstream DNA and the downstream nucleosome, becoming a separator.

As for SHL(−1), classification yielded three EC-nucleosome structures: SHL(−1), SHL(−1)_{+1A}, and SHL(−1)_{+1B} (Fig. 2A and figs. S5 and S6). According to the EC progression, the nucleosome rotates on the downstream DNA axis in front of RNAPII (~36°/bp because of the DNA helical pitch). Judging from the RNAPII-nucleosome distances and the nucleosome rotation angles, the ECs in SHL(−1)_{+1A} and SHL(−1)_{+1B} are advanced downstream by 1 bp with no obvious change in the histone-DNA contacts, relative to the SHL(−1) complex. The SHL(−1)_{+1A} and SHL(−1)_{+1B} complexes differ in their nucleosome orientations. These three structures may reflect the nucleosome mobility ahead of RNAPII (movie S1). As compared with the SHL(−1) complex without elongation factors (6), the nucleosome in the current SHL(−1) complex is rotated around the downstream DNA axis, and shifted away from the Rpb2 lobe, to avoid steric clashes with Elf1 and Spt5 NGN (Fig. 2A, and figs. S12, A to D, and S13). This direction of the nucleosome rotation is consistent with the forward EC translocation, suggesting that the elongation factors may help EC shift forward.

In the SHL(−1), SHL(−1)_{+1A}, and SHL(−1)_{+1B} complexes, the DNA is torn off from one of the

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two H2A-H2B dimers and its adjacent H3-H4, but the EC retains the intact histone octamer (Fig. 1D and fig. S6). Elf1 is located at the pivot point of the nucleosome rotation around the downstream DNA axis, and the Elf1 β sheet (the $\beta 3$ and $\beta 4$ strands) directly contacts the DNA-peeled parts of H3-H4 in SHL(-1) and SHL(-1)_{+1A} (Fig. 2, B to D, and fig. S12E). The NGN domain of Spt5 is close to the nucleosomal DNA [SHL(-1)] or H2A-H2B [SHL(-1)_{+1A} and SHL(-1)_{+1B}]. Thus, Elf1 and Spt4/5 serve as a separator between the downstream DNA and the nucleosome, preventing the DNA reassociation to the exposed histones. The histone-contacting amino-acid residues in Elf1 $\beta 3$ and $\beta 4$ are not well conserved, and their mutations do not substantially affect nucleosome transcription (fig. S14). The absence of specific interactions may be favorable for the nucleosome rotation in front of RNAPII.

In SHL(-1)_{+1B}, the nucleosome is brought in closer proximity to RNAPII than that in SHL(-1)₊₁ without elongation factors (Fig. 2E). Specifically, the Rpb1 clamp head is adjusted at the contact site between the DNA and H3-H4 to act as a “wedge” between them (Fig. 2F and fig. S12F). This is not the case in the absence of the elongation factors (Fig. 2G). Moreover, the conserved N-terminal basic tail of Elf1 could interact with the DNA near the histone-DNA contact site and may compete with H3-H4 for the DNA to facilitate the separation of the histone-DNA contact (Fig. 2C). The deletion of the Elf1 N-terminal tail impaired the transcription processivity beyond SHL(-1) (Fig. 2H). Thus, Elf1 helps dissociation of the histone-DNA contact, and this is favorable for the progression of the RNAPII wedge upon separation of the contact.

At SHL(-1), the DNA-peeled parts of the histones tend to associate with another DNA segment (a “foreign” DNA) to generate a nucleosome-like structure (6), which could become an intermediate for the cis or trans histone transfer to other DNA regions and might perturb the chromatin structure and epigenetic information. However, the foreign DNA binding was strongly suppressed in the presence of the elongation factors. Classifications revealed that the binding of the foreign DNA and Elf1 is virtually mutually exclusive (figs. S5 and S6). Elf1 contacts the DNA-peeled H3-H4 (Fig. 2C), thus blocking the foreign DNA binding. In addition, although they are disordered, Elf1 and Spt5 have a C-terminal acidic tail and an N-terminal acidic tail, respectively, which could cover and hold the exposed H2A-H2B (Fig. 2C and fig. S12G). Spt16 (FACT) also conserves an acidic tail (fig. S15), which interacts with H2A-H2B (27). These findings are consistent with the previous observations that Elf1 and Spt4/5 are implicated in the chromatin structure maintenance (20, 21). Because Elf1 and Spt4/5 are genetically or physically linked to histone chaperones Spt6 and FACT, they might cooperate for the nucleosome reassembly in the wake of the EC passage.

In the SHL(-5) complex, the downstream DNA is slightly bent because of the Elf1 intervention, and the nucleosome is located more

distant from RNAPII, as compared with those in the previous SHL(-5) complex without the elongation factors (Fig. 3) (6). In the absence of the elongation factors, the nucleosome is trapped between the Rpb1 clamp head and the Rpb2 lobe (6, 28). By contrast, in the presence of the elongation factors, Elf1 occupies the same place, and consequently, the RNAPII-nucleosome contacts are lost. The nucleosomal DNA is ~ 7 Å apart from Elf1, and there is no apparent EC-nucleosome interaction that could trap the nucleosome (fig. S16A). Consistently, modeling of a 1-bp advanced EC [SHL(-5)₊₁] suggests that the

nucleosome can rotate in front of the EC without clashing with the Rpb1 clamp head, whereas there is a steric clash in the absence of the elongation factors (fig. S16B). Thus, the elongation factors minimize the RNAPII-nucleosome interaction at SHL(-5) and prevent the EC from being trapped in a stable paused state. Similar mechanisms may be applicable to the suppression of the SHL(-6) and SHL(-2) barriers because the nucleosome is trapped between the Rpb1 clamp head and the Rpb2 lobe in the absence of elongation factors (6) and sterically incompatible with Elf1 and/or Spt4/5 (fig. S16C).

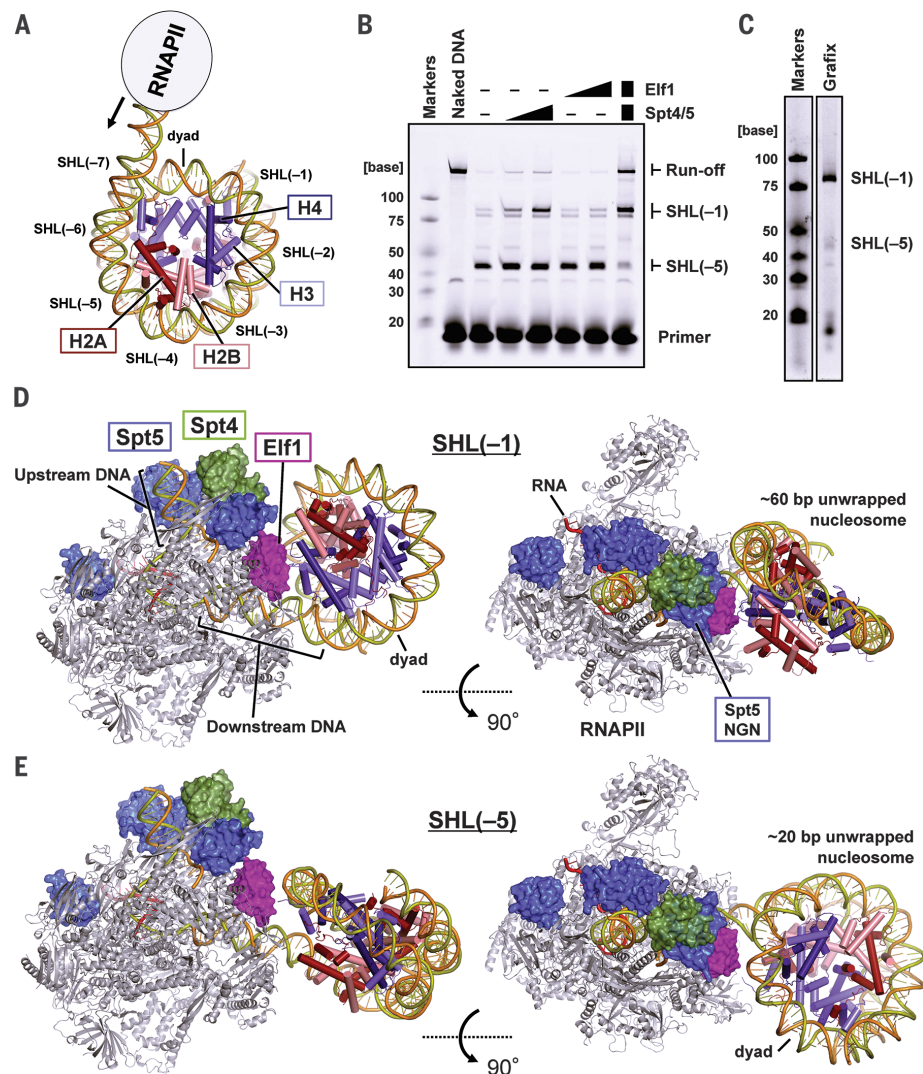


Fig. 1. Cryo-EM analyses of the EC-nucleosome complexes. (A) The experimental setup. The template DNA is colored yellow, and the nontemplate DNA is colored orange. Histones H2A, H2B, H3, and H4 are colored dark red, light red, light blue, and dark blue, respectively. (B) The effects of Elf1 and Spt4/5 on nucleosomal transcription. The elongated RNAs were analyzed by means of urea polyacrylamide gel electrophoresis (PAGE). The concentrations of Spt4/5 are 0.1 and 0.4 μ M, and those of Elf1 are 0.25 and 1.0 μ M. The experiment was performed in triplicate. (C) Urea PAGE of the sample used for the cryo-EM analyses, after purification by Grafix. (D) Cryo-EM structure of the EC-nucleosome complex at SHL(-1). RNAPII is shown as a gray cartoon model. Elf1, Spt4, and Spt5 are shown with magenta, green, and blue surfaces, respectively. (E) Cryo-EM structure of the EC-nucleosome complex at SHL(-5).

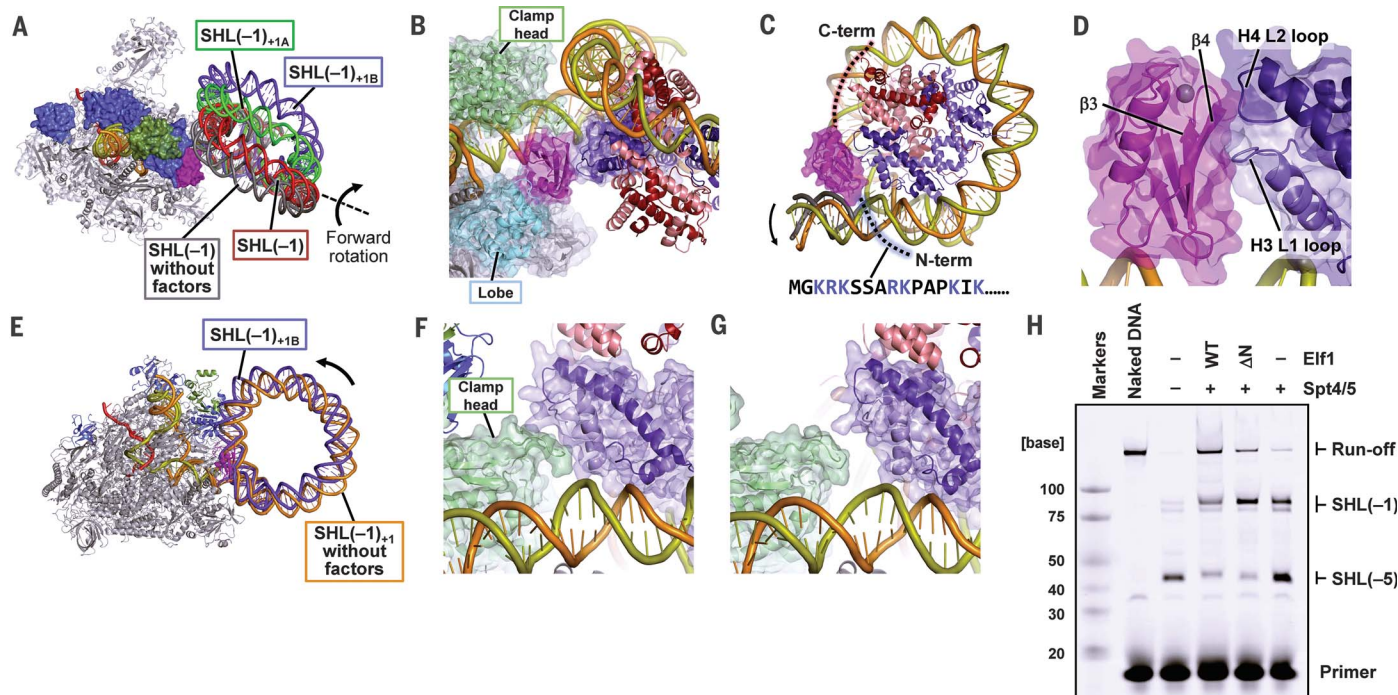


Fig. 2. The EC-nucleosome interactions at SHL(-1). (A) The EC-nucleosome structures at SHL(-1) (red), SHL(-1)_{1A} (green), SHL(-1)_{1E} (blue), and the RNAPII-nucleosome complex at SHL(-1) (PDB 6A5T; gray) are superimposed in terms of RNAPII. The EC is colored as in Fig. 1. (B) Close-up view of the EC-nucleosome interface at SHL(-1). RNAPII, Elf1, and H3-H4 are overlaid with surface representations. The Rpb1 clamp and the Rpb2 lobe are colored pale green and cyan, respectively. Spt4/5 is omitted for clarity. (C) Elongation factor-induced bending of the downstream DNA. The peeled DNA in the absence of elongation factors (PDB 6A5V) is colored gray. The Elf1 N- and C-terminal tails are indicated

as dotted lines. **(D)** Close-up view of the Elf1-histone interface. The Zn^{2+} ion bound to Elf1 is shown as a gray sphere. **(E)** Elongation factor-induced shift of the nucleosome. The nucleosomal DNA in the SHL(-1)_{+1B} complex is colored blue, and that in the absence of elongation factors [SHL(-1)₊₁, PDB 6A5V] is colored orange. **(F)** The clamp head-nucleosome interaction in the SHL(-1)_{+1B} complex. Elf1 is omitted for clarity. **(G)** The same view as in (F) in the absence of elongation factors (PDB 6A5V). **(H)** An assay analyzing the effect of the Elf1 N-terminal tail on the nucleosomal transcription. The concentrations of Spt4/5 and Elf1 are 0.4 and 1.0 μM , respectively.

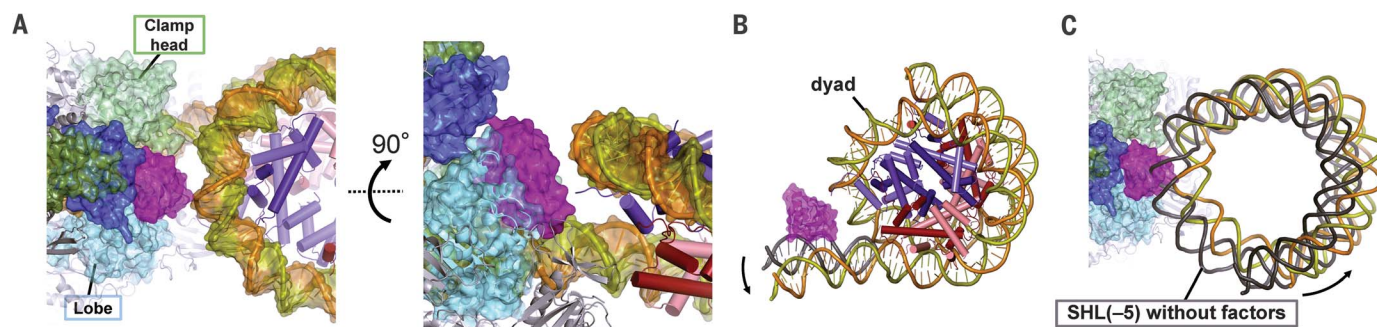


Fig. 3. The EC-nucleosome interactions at SHL(−5). (A) Close-up views of the EC-nucleosome boundary at SHL(−5). Colors are as in Fig. 2B. The EC and the nucleosomal DNA are overlaid with surface representations, showing a gap between them. (B) Elongation factor-induced bending of the downstream DNA. The nucleosome in the SHL(−5) complex is

superimposed with that, without elongation factors (colored gray; PDB 6A5P). **(C)** Elongation factor-induced shift of the nucleosome. The SHL(-5) structure is superimposed with that, without elongation factors (PDB 6A5P) in terms of RNAPII. The nucleosomal DNA in the latter complex is colored gray.

We elucidated how the conserved elongation factors facilitate efficient RNAPII passage through a nucleosome (Fig. 4). This may better represent the transcription of gene-body nucleosomes because Spt4/5 and Elfl associate with elongating RNAPII (17). In the cellular context, EC cooperates with the other elongation factors, histone

chaperones, and nucleosome remodelers, which could help relieve the nucleosomal barriers. The observed partially DNA-peeled nucleosome at SHL(-1) or SHL(-5) should provide a platform for various chromatin functions associated with these factors. By contrast, the promoter-proximal, first (+1) nucleosome serves as a strong transcrip-

tional barrier in many eukaryotic genes (5, 29). In this case, RNAPII stalls owing to DNA-binding factors or negative factors probably near the entry of the nucleosome, and this paused complex differs from that involved in gene-body transcription (30). Further studies will clarify the mechanism of the promoter-proximal regulation.

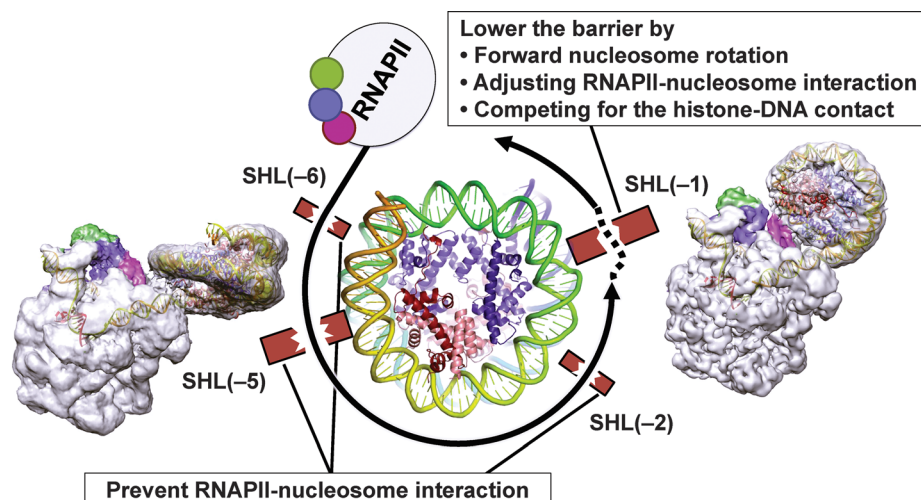


Fig. 4. Roles of Elf1 and Spt4/5 in the nucleosome transcription. Elf1 and Spt4/5 modify the RNAPII surface to intervene with the RNAPII-nucleosome interaction. At SHL(−6), SHL(−5), and SHL(−2), the elongation factors facilitate the EC passage by preventing the direct RNAPII-nucleosome interaction that causes pausing. They facilitate the EC passage through SHL(−1), not only by changing the RNAPII-nucleosome interactions to adjust the RNAPII wedge to the histone-DNA contact site but also by weakening the histone-DNA contacts via the Elf1 basic tail.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/744/suppl/DC1
Materials and Methods
Figs. S1 to S16
Tables S1 and S2
References (31–40)
Movie S1

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BCR-dependent lineage plasticity in mature B cells

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B2 cells engage in classical antibody responses, whereas B1 cells are considered carriers of innate immunity, biased toward recognizing epitopes present on the surfaces of common pathogens and self antigens. To explore the role of B cell antigen receptor (BCR) specificity in driving B1 cell differentiation, we developed a transgenic system allowing us to change BCR specificity in B cells in an inducible and programmed manner. Mature B2 cells differentiated into bona fide B1 cells upon acquisition of a B1 cell–typical self-reactive BCR through a phase of proliferative expansion. Thus, B2 cells have B1 cell differentiation potential in addition to their classical capacity to differentiate into memory and plasma cells, and B1 differentiation can be instructed by BCR-mediated self-reactivity, in the absence of B1-lineage precommitment.

B cells are the carriers of humoral immunity. On the basis of their phenotype and functional properties, mature B cells can be subdivided into B1 and B2 cells, with the former further divided into the CD5⁺ B1a and CD5[−] B1b subsets. B2 cells form follicles in the spleen and lymph nodes and are responsible for generating specific antibody responses against foreign antigens, typically involving T cell–dependent affinity maturation and somatic hypermutation of the B cell antigen receptor (BCR) (1). In contrast, B1 cells are predominantly found in peritoneal and pleural cavities and produce natural antibodies, providing a “first line of defense” against common bacterial pathogens and contributing to the clearance of apoptotic cells and oxidized lipids (2, 3). Therefore, B1 cells are thought to perform innate-like functions in the immune system, predominantly expressing evolutionarily selected BCRs with low levels of somatic mutation and functional diversity (4–6).

Despite major advances in the field, the origin of B1 cells remains controversial. The “lineage

model” posits that B1 and B2 cells represent separate lineages that arise from different progenitor populations, committed to either lineage prior to BCR expression. In the case of B1 cells, this possibly even occurs prior to hematopoietic stem cell differentiation (7–10). As recently reviewed in detail, definitive evidence for the lineage model is still lacking, with both putative BCR-negative B1 progenitors and exclusive progenitor commitment to B2 differentiation being controversial (11, 12). In the “selection model,” B cell progenitors are instructed by BCR specificity to differentiate into B1 cells, depending on BCR-mediated recognition of common bacterial and certain self antigens (2, 13). Although strong evidence indeed indicates that certain BCR specificities can exclusively drive B1 cell differentiation, this does not rule out a possible commitment of progenitor cells to either lineage prior to BCR expression (14). However, as demonstrated below, the existence of B1 and B2 cell–typical BCRs can be exploited to test lineage commitment and its control by BCR specificity in mature B cells.

To address this issue, we generated a genetic system that would allow a BCR-dependent interconversion of mature B1 and B2 cells. For this purpose, we targeted two prearranged immunoglobulin heavy (IgH) chain variable (V) region gene segments—V_H12 and B1-8, respectively—in a head-to-head orientation and flanked by inverted loxP sites into the J_H region of the IgH locus. Cre-mediated recombination results in inversion of this V_H12B1-8^{fl} cassette, leading to a situation in which some cells express B1-8 and others V_H12 IgH chains. In combination with V_κ4 Ig light chains, the V_H12 and B1-8 IgH chains form B1 and B2 cell–typical BCRs, respectively (14, 15). V_H12B1-8^{fl} mice (hereafter termed B2 mice) and B1-8V_H12^{fl} mice (hereafter termed B1 mice) should predominantly develop B cells with either B1-8/V_κ4 or V_H12/V_κ4 BCRs (Fig. 1A). In agreement with previous work, the knocked-in BCRs allowed the developing cells to circumvent the pro/pre-B cell stage (fig. S1, A to E) (15). When the B cells in these animals were stained

with the BCR-specific anti-idiotypic antibodies Acl46 and 5C5, respectively (14, 16), essentially all splenic B cells of B2 mice were Acl46⁺ and of the B2 cell phenotype, characterized by their small size and a CD19⁺, B220⁺, IgD^{hi}, CD5[−], CD23^{+/lo}, and CD43[−] surface phenotype. In contrast, B cells of B1 mice were 5C5⁺ and acquired the B1 cell phenotype, characterized by large cell size and a CD19^{hi}, B220^{lo}, IgD^{lo}, CD5⁺, CD23[−], and CD43⁺ surface phenotype (Fig. 1B). This is consistent with the natural expression of this BCR that is specific for phosphatidylcholine (PtC) and exclusively expressed on B1 cells of wild-type mice (13) (fig. S1D). PtC is a common membrane phospholipid and is exposed on senescent red blood cells, which suggests that V_H12/V_κ4 antibodies are involved in the clearance of these cells (17, 18). When splenic B cells of B1 and B2 mice were isolated and transduced with TAT-Cre in vitro, approximately 10% of these cells inverted the IgH insertion cassette (fig. S1G). This led to detectable populations of cells that switched from the expression of the original BCR to that of the new BCR, thereby confirming the functionality of the transgenic system (Fig. 1C and fig. S1F). For simplicity, we refer to B1-8V_H12^{fl} and V_H12B1-8^{fl}–derived switched B cells as B1→B2 and B2→B1 cells, respectively.

To test whether a change of BCR specificity would change the phenotype of fully mature B cells, we treated B1 and B2 mice for 2 weeks with antibodies against the interleukin-7 receptor (IL-7R) to block the influx of immature B cells into the spleen (fig. S2). Purified splenic B cells from these mice were then transduced with TAT-Cre in vitro. Only very low numbers of B1→B2 cells developed. In contrast, B2→B1 cells developed into a dominant population (Fig. 1C and fig. S1F). However, although these cells acquired some B1-typical markers, their phenotypic change was only partial in vitro (fig. S3). We therefore transferred IgM^a experimental (TAT-Cre transduced) mature B cells together with IgM^b carrier B cells into immunodeficient recipients to study the consequences of the BCR switch in a more physiological environment (Fig. 2A) (19). Consistent with the in vitro data, only low numbers of B1→B2 cells were recovered from the recipients. These were located predominantly in the peritoneal cavity and had acquired a phenotype intermediate between B1 and B2 (fig. S4). In contrast, B2→B1 cells expressing the 5C5 idiotype were readily detectable in the spleen and peritoneal cavity of the recipients 4, 8, and 30 days after transfer and became the dominant population among the IgM^a experimental cells (Fig. 2B). Contributing to this dominance was an initial, transient phase of rapid proliferation, similar to the clonal expansion of PtC-specific B1 cells observed in wild-type mice over the first 3 to 4 weeks of life (Fig. 2C and fig. S5A) (4, 20). Strikingly, after completion of this expansion phase, the B2→B1 cells became resting cells that phenotypically were essentially indistinguishable from bona fide B1 cells. B2→B1 cells reproducibly increased in size, down-regulated B220 and IgD, lost CD23 and CD21 expression, and increased

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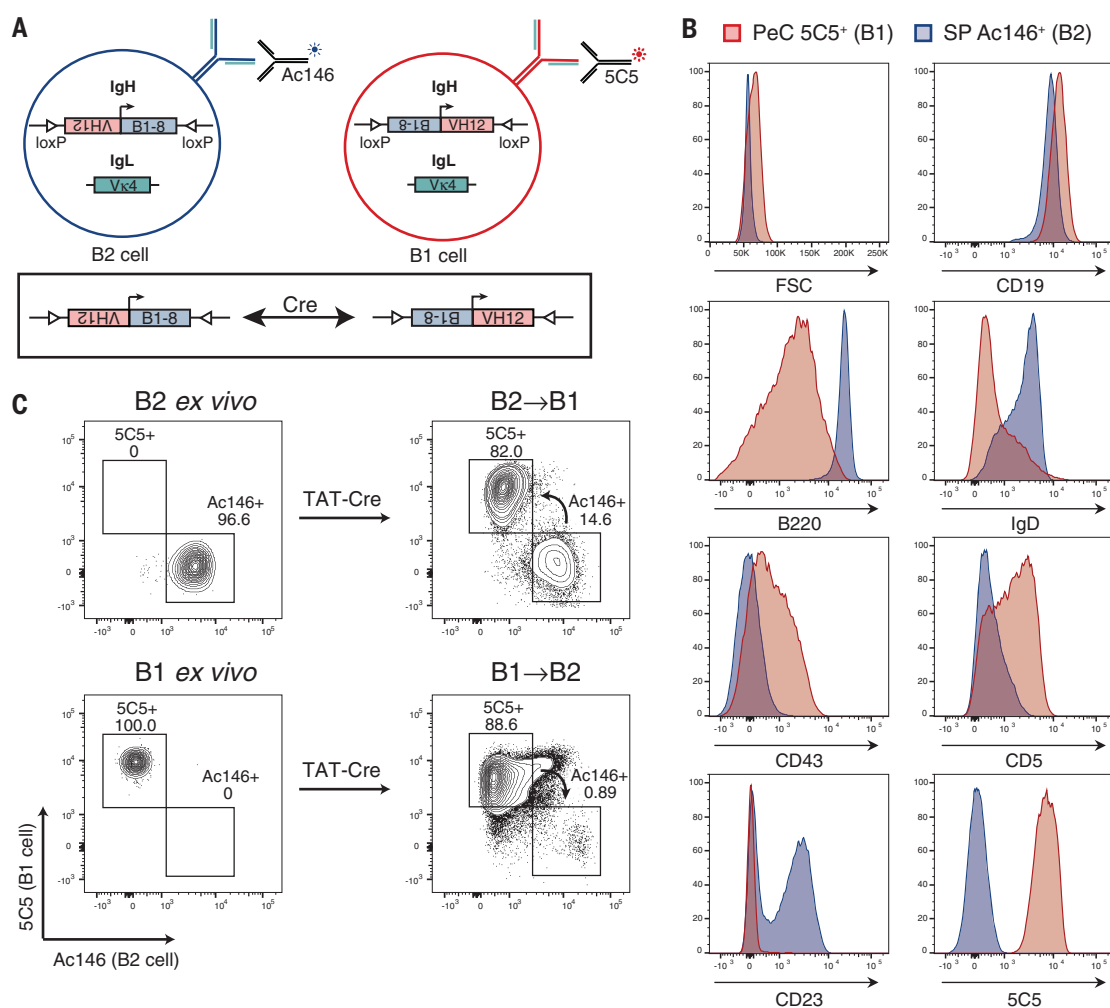
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Fig. 1. Genetic system to switch BCR specificity.

(A) Schematic of Cre-mediated switch of BCR specificity. Mice with the IgH cassette in the $V_H12B1-8^{fl}$ orientation (B2 mice) develop B2 cells with the B1-8/ $V_{\kappa}4$ BCR (left), whereas mice with the B1-8/ V_H12 orientation (B1 mice) develop B1 cells with the $V_H12/V_{\kappa}4$ BCR (right). The flanking inverted loxP sites (bottom) allow the orientation of the cassette, and thus BCR specificity, to be changed by application of Cre.

(B) Cell surface phenotype of splenic $Ac146^+$ B cells from B2 mice (blue) and peritoneal $5C5^+$ B cells isolated from B1 mice (red) measured by flow cytometry. FSC, forward scatter (area). (C) BCR expression via flow cytometry on B cells isolated from spleens of B2 mice (top) or B1 mice (bottom), 4 days after treatment with TAT-Cre *in vitro*. B1-8 and V_H12 were detected by the anti-idiotypic antibodies $Ac146$ and $5C5$, respectively. Data are representative of 10 to 20 independent experiments.



the expression of CD19, CD43, and CD5 (Fig. 2, D and E, and fig. S5B). In the case of the B1a-specific marker CD5, the up-regulation was modest in splenic and not significant in peritoneal B2→B1 cells. This may relate to the observation that adult bone marrow-derived B1a cells express lower levels of CD5 than fetal liver-derived B1a cells (21, 22).

The B2→B1 conversion was not dependent on B2 cell subtype or donor organ, as marginal zone and spleen- or lymph node-derived follicular B2 cells were all able to acquire the B1 cell phenotype (fig. S6). Consistent with this, we calculated that at least ~40% of the splenic B cells that successfully inverted the $V_H12B1-8^{fl}$ allele assumed a B1 cell phenotype, excluding the possibility that the B2→B1 cells developed from some small cellular subset (fig. S7 and table S1). To control for potential biases caused by (unlikely) endogenous IgL gene rearrangements, all experiments on B2→B1 conversion were also performed using Rag2-deficient donor B2 mice, with identical results (see supplementary materials). Furthermore, to exclude that the B2→B1 conversion was peculiar to the *in vitro* treatment of these cells with bacterial-derived TAT-Cre

protein (with its possible endotoxin contamination), we crossed the tamoxifen-inducible conditional R26CreER^{T2} allele into the donor B2 mice (23). The recipients of B cells from these mice were then administered tamoxifen 1 day after transfer. As in the case of TAT-Cre, B2→B1 cells developed and adopted the B1 cell phenotype (Fig. 2E and fig. S8). In the course of these experiments, we noticed that the donor B2 mice contained minute numbers of spontaneously arising IgM^a $5C5^+$ cells in the spleen and a dominant population of these cells in the peritoneal cavity (fig. S9). This was likely a consequence of rare spontaneous recombination events between the inverted loxP sites, as has been previously reported, followed by positive selection and clonal expansion (24). The frequency of this population was increased in the presence of the R26CreER^{T2} allele (fig. S9). To exclude the possibility that these cellular contaminants contributed to B2→B1 cell development in the recipients (even though they should have been eliminated by the B cell purification procedure), we performed cell transfer experiments without the delivery or induction of Cre. In this situation, IgM^a $5C5^+$ cells were undetectable in the recipients, validating our ex-

perimental approach (fig. S9). Thus, upon expression of a B1-typical BCR, mature B2 cells acquire the phenotype of B1 cells and persist as such *in vivo*.

The phenotypic change of B2→B1 cells suggested that these cells might also adopt B1-typical functions. B1 cells home to the peritoneal cavity and secrete natural antibodies into the blood. In the B2→B1 recipient animals, the percentages of the experimental IgM^a B cells were markedly increased in the peritoneal cavity relative to the spleen. Moreover, essentially all of these peritoneal IgM^a B cells were $5C5^+$ B2→B1 cells, indicating that these cells homed more efficiently to this anatomical site than did the IgM^b carrier B cells or the nonswitched $Ac146^+$ B cells, similar to natural B1 cells (Fig. 3, A and B). B2→B1 cells were also present in the pleural cavity, the second natural niche of B1 cells (Fig. 3C). Consistent with the spontaneous production of antibodies by B1 cells, $5C5$ -binding IgM^a-positive antibodies were detectable in the circulation of the recipients (Fig. 3D). B1 and B2 cells are also known to have different functional properties when cultured *in vitro*. Relative to B2 cells, B1 cells show prolonged survival *in vitro*

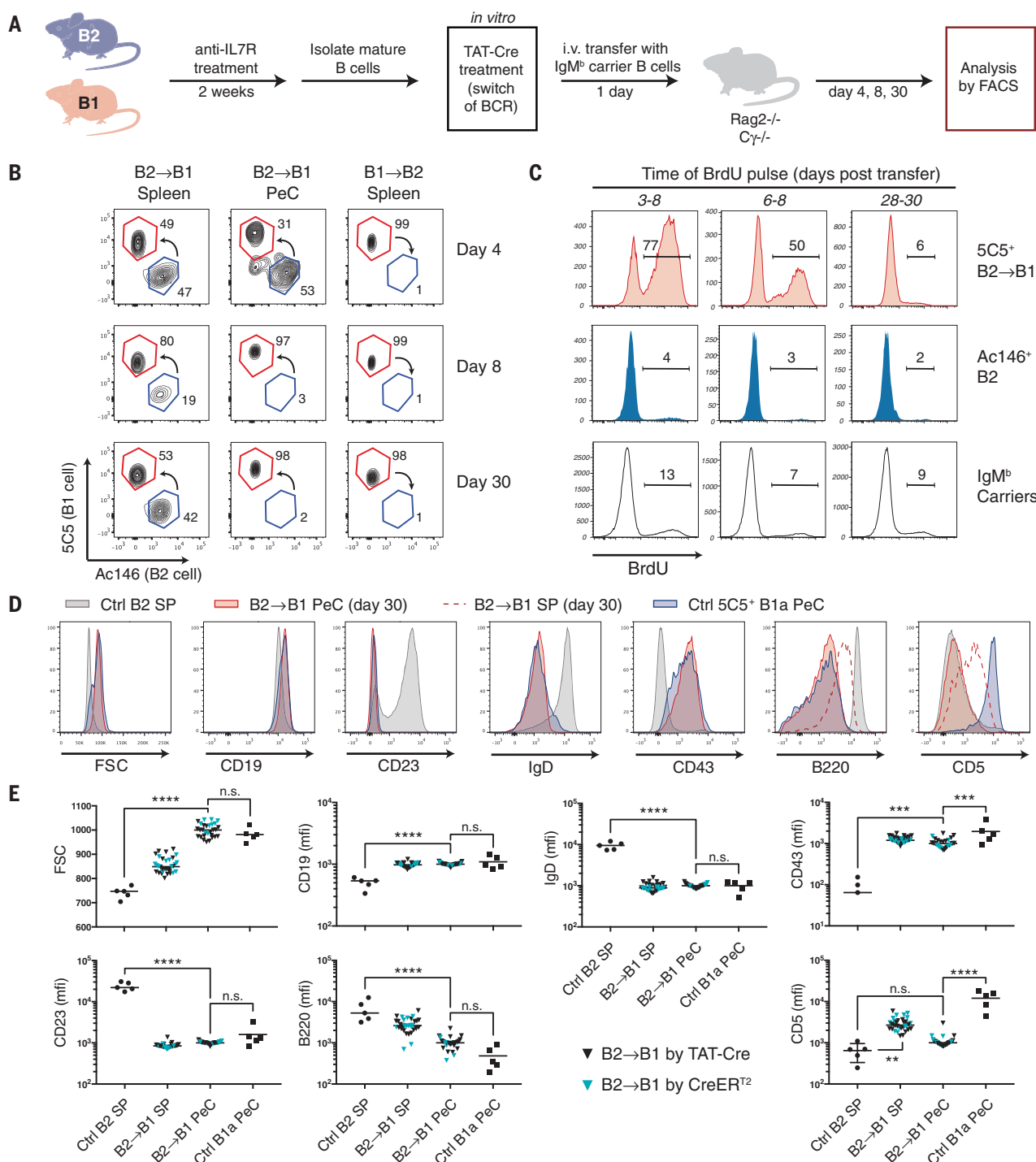


Fig. 2. B2→B1 cells acquire the surface phenotype of B1 cells and persist in vivo after a phase of proliferative expansion. (A) Experimental scheme: B1 and B2 mice were treated for 2 weeks with antibodies to IL-7R to block B cell development. Splenic B cells were then treated with TAT-Cre *in vitro* and transferred intravenously to immunodeficient recipients the next day. FACS, fluorescence-activated cell sorting. (B) BCR expression via flow cytometry on experimental IgM^B B cells isolated from the indicated anatomical sites at the indicated time points after transfer. (C) Proliferation analysis based on bromodeoxyuridine (BrdU). Animals were fed with BrdU for the indicated time windows. The BrdU content of the indicated cells was analyzed on day 8 (left and center) or day 30 (right) by flow cytometry. (D) Flow cytometric analysis of cell surface phenotype of peritoneal B2→B1

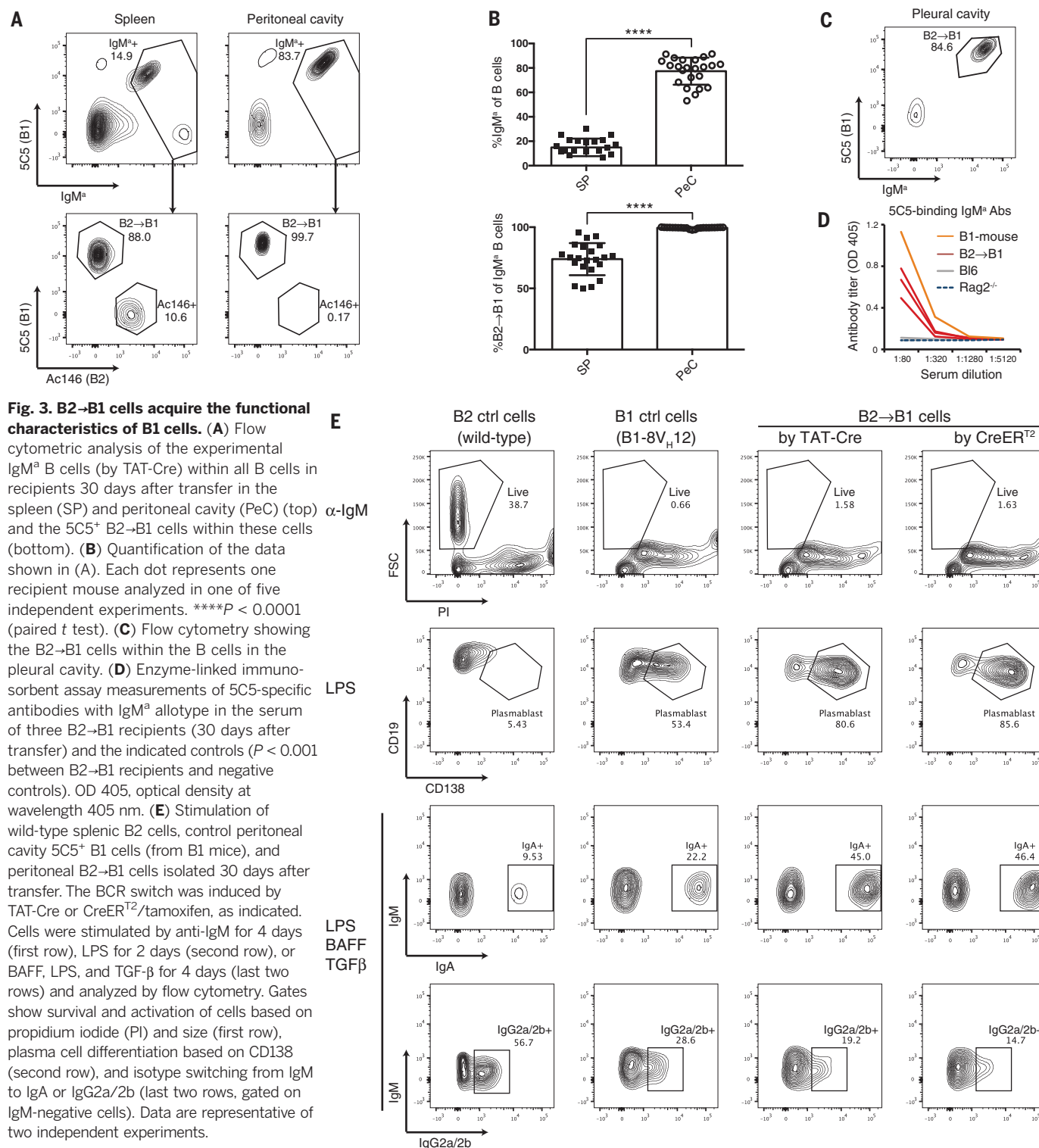
cells (red) isolated from the recipients 30 days after transfer, and of control splenic B2 (gray) and peritoneal (5C5⁺) B1a cells (blue) isolated from wild-type animals. Splenic B2→B1 cells (dashed red curves) are only shown for the markers in which they differ relative to peritoneal B2→B1 cells [see (E)]. (E) Summary of the B2→B1 cell surface phenotype from five independent experiments. Each dot represents the median fluorescence intensity (mfi) of the indicated surface marker and cell population of one recipient mouse or control mouse, normalized to the median mfi in PeC B2→B1 cells (set to 1000) per experiment. Tamoxifen/CreER^{T2}-derived B2→B1 cells are colored turquoise (see fig. S7 for details). ****P < 0.0001, ***P < 0.001, **P < 0.01 (ordinary one-way analysis of variance and Bonferroni multiple-comparisons test); n.s., not significant.

in the absence of stimulation and faster differentiation into CD138⁺ plasma cells upon lipopolysaccharide (LPS) stimulation (25, 26). In addition, unlike B2 cells, they are refractory to anti-IgM F(ab)₂-induced BCR engagement and show efficient class switching to IgA upon stimulation with B cell-activating factor (BAFF), LPS, and transforming growth factor- β (TGF- β)

(27–29). Without exception, the response of peritoneal B2 $\rightarrow$ B1 cells to these stimuli was similar to that of B1 cells (Fig. 3E and fig. S10). Thus, B2 $\rightarrow$ B1 cells not only acquired the B1 cell surface phenotype, but also adopted B1-typical functional properties *in vitro* and *in vivo*.

To expand the analysis of the identity of B2 $\rightarrow$ B1 cells beyond the limited number of sur-

face markers, we performed RNA sequencing (RNA-seq) of peritoneal B2 $\rightarrow$ B1 cells and compared their transcriptome to that of peritoneal B1 and splenic B2 control cells. In terms of a previously defined B1 signature consisting of 14 B1-specific and 14 B2-specific genes (30), B2 $\rightarrow$ B1 cells showed an expression profile almost identical to that of B1 control cells (Fig. 4A). This



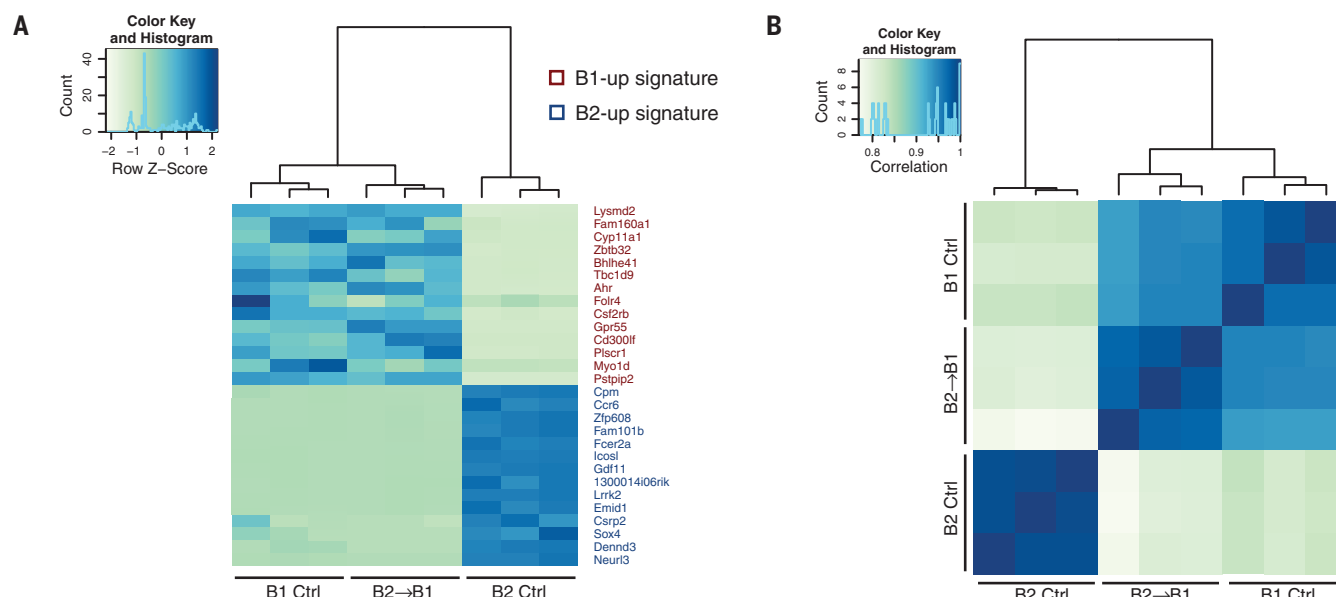


Fig. 4. B2→B1 cells acquire the transcriptome profile of B1 cells.

(A) Heat map with sample clustering of gene expression levels of 14 genes specifically expressed in B1 cells (red) and 14 genes specifically expressed in B2 cells (blue) previously defined as B1 signature (30). RNA was isolated from splenic control B2 cells from B2 mice (B2 Ctrl), peritoneal 5C5⁺ cells

from B1 mice (B1 Ctrl), and B2→B1 cells isolated from the peritoneal cavity of recipients 30 days after transfer (B2→B1). (B) Heat map of Spearman correlations between each sample, based on the expression levels of 7629 expressed genes. Each B2→B1 sample is based on one donor animal and was pooled from three or four recipients.

result was further confirmed by a transcriptome-wide analysis, where the Spearman correlations between B2→B1 and B1 control samples were as high as those between biological replicates and significantly higher than those between B2→B1 and B2 control samples, closely clustering B1 and B2→B1 samples together (Fig. 4B). Thus, the B2→B1 conversion entails profound genetic alterations, leading to a B1-typical transcriptome profile in B2→B1 cells.

Taken together, mature B2 cells of the various subsets exhibit plasticity toward B1 cell differentiation. The cells undergo B1 differentiation upon substitution of their BCR by an autoreactive BCR that is known to drive B1 cell development in early postnatal life. In both instances, differentiation is initially linked to extensive cell proliferation, likely reflecting a special mode of B cell activation by autoantigen (4, 20). The present experimental system offers a unique opportunity to study this process, which is key to the understanding of BCR repertoire selection in the B1 compartment. In contrast, only low numbers of B1 cells switching to the expression of the B2-typical BCR were recovered from the animals, with a surface phenotype intermediate between B1 and B2. This is likely a consequence of the fact that B1 cells represent B cells that have undergone antigen-driven activation, proliferation, and differentiation. The profound genetic and morphological changes that the cells undergo during these processes may well be irreversible.

Jerne's notion that autoreactive receptors initially drive lymphocyte development, with the subsequent selection of escape somatic mutants, was accommodated in the classical model of B cell development through ordered V(D)J gene rearrangements, in first the IgH and then the IgL

loci of progenitor B cells (1, 31, 32). In this model, IgH chain-containing pre-BCRs constitutively drive clonal expansion sustained by the surrogate light chain, followed by sequential IgL chain gene rearrangements and IgL chain editing. Although these ordered gene rearrangements clearly dominate postnatal B cell development, recent evidence indicates that during fetal development, very much in agreement with Jerne, IgH and IgL chains are often initially coexpressed (33). In this situation, autoreactive BCRs instead of pre-BCRs drive the expansion and selection of B cells, which at that developmental stage predominantly develop into the B1 variety (11, 34).

Although the control of these different pathways of differentiation remains to be fully elucidated, the present results establish that mature B2 cells are not irreversibly committed to a B2 "lineage" but maintain the potential to develop into B1 cells. That these cells do so upon engraftment of an autoreactive, B1 cell-typical BCR demonstrates the instructive role of BCR specificity and mode of signaling in driving B1 cell differentiation, in the absence of B1-lineage precommitment.

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provided scientific and experimental advice; R.G. and K.R. wrote the manuscript; and K.R. supervised the project.

Competing interests: The authors declare no competing financial interest. **Data and materials availability:** The RNA-seq data are available in the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo) under accession number GSE124827. The materials required to use the transgenic system described here will be available through K.R. under a material transfer agreement from the MDC.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6428/748/suppl/DC1
Materials and Methods
Figs. S1 to S10
Table S1
References (35–40)

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STRUCTURAL BIOLOGY

Structural insight into substrate and inhibitor discrimination by human P-glycoprotein

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ABCB1, also known as P-glycoprotein, actively extrudes xenobiotic compounds across the plasma membrane of diverse cells, which contributes to cellular drug resistance and interferes with therapeutic drug delivery. We determined the 3.5-angstrom cryo-electron microscopy structure of substrate-bound human ABCB1 reconstituted in lipidic nanodiscs, revealing a single molecule of the chemotherapeutic compound paclitaxel (Taxol) bound in a central, occluded pocket. A second structure of inhibited, human-mouse chimeric ABCB1 revealed two molecules of zosuquidar occupying the same drug-binding pocket. Minor structural differences between substrate- and inhibitor-bound ABCB1 sites are amplified toward the nucleotide-binding domains (NBDs), revealing how the plasticity of the drug-binding site controls the dynamics of the adenosine triphosphate-hydrolyzing NBDs. Ordered cholesterol and phospholipid molecules suggest how the membrane modulates the conformational changes associated with drug binding and transport.

ABCB1, or P-glycoprotein, is an ATP-binding cassette (ABC) transporter of physiological and clinical importance. Its nucleotide-binding domains (NBDs) harness the energy of ATP hydrolysis to generate conformational changes in the transmembrane domains (TMDs) that facilitate the shuttling of chemically diverse compounds across many blood-organ barriers (1–4). Consequently, ABCB1 activity can confer multidrug resistance to cancer cells and prevent drugs from reaching therapeutic con-

centrations in target cells or organs, which complicates chemotherapy and/or the treatment of certain neurological disorders. Despite showing promise in model systems (5–7), chemosensitization of multidrug-resistant cells through the simultaneous delivery of ABCB1 inhibitors (e.g., the third-generation inhibitor zosuquidar) and chemotherapeutic drugs (e.g., Taxol/paclitaxel) has so far been clinically unsuccessful (8, 9). To understand the interaction of ABCB1 with small-molecule compounds, to rationalize its substrate

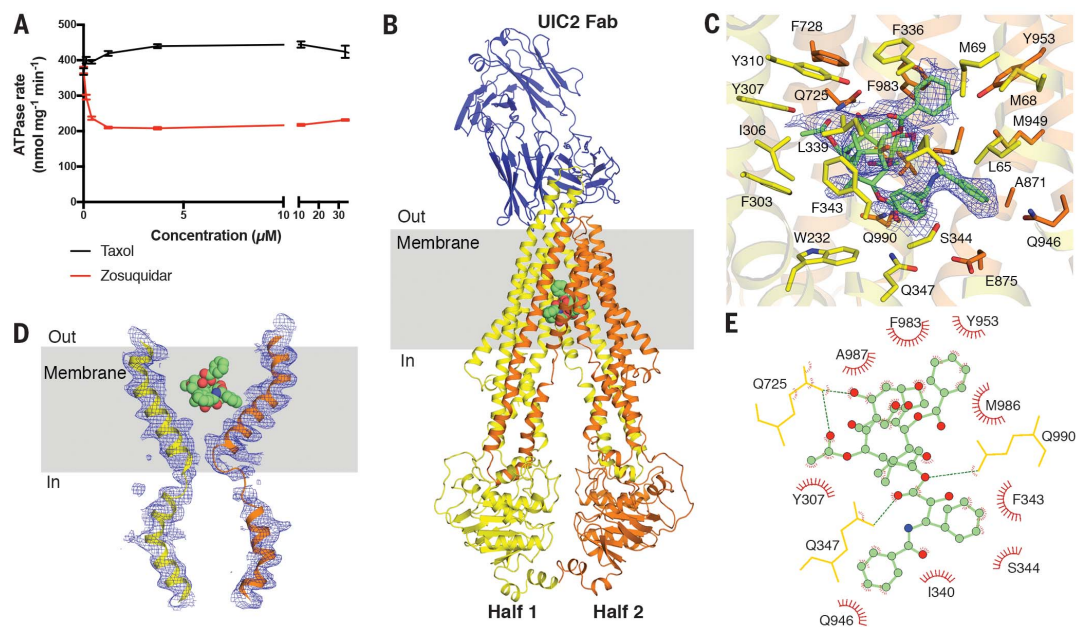
specificity and the discrimination of substrates and inhibitors, and to facilitate the development of more specific or potent inhibitors for clinical use, structural insight into drug and inhibitor binding to ABCB1 is essential. No structures of ABCB1 bound to transport substrates are available at present, and inhibitor-bound and apo structures are only available for detergent-solubilized ABCB1 and remain controversial because proper ABCB1 function is strongly dependent on the membrane.

We reconstituted ABCB1 in nanodiscs comprising a mixture of brain polar lipids and cholesterol and determined near-atomic resolution cryo-electron microscopy (cryo-EM) structures in complex with Taxol (3.6-Å resolution) or zosuquidar (3.9-Å resolution). In both cases, the antigen-binding fragment (Fab) of the inhibitory antibody UIC2 (10), shown to be compatible with inward-open and occluded conformations (11), was added (complex mass: ~200 kDa) to facilitate higher-resolution structure determination.

Nanodisc-reconstituted wild-type human ABCB1 (ABCB1_H) displayed ATPase activity in the range of 200 to 400 nmol ATP mg⁻¹ min⁻¹, which was mildly stimulated by Taxol and inhibited by zosuquidar (Fig. 1A), in agreement with earlier observations (12, 13). This suggested that at 10 μM, the Taxol concentration chosen for structural studies, a sufficiently large fraction of ABCB1_H molecules should contain bound drug.

Fig. 1. In vitro function and structure of nanodisc-reconstituted ABCB1.

(A) Taxol- and zosuquidar-modulated ATPase activity. Data points analyzed represent means of three independent measurements. Error bars indicate SD. (B) Ribbon diagram of human ABCB1 bound to Taxol (green spheres). The N- and C-terminal halves of ABCB1 are colored yellow and orange, respectively, with the UIC2 Fab shown in blue. (C) Close-up of binding site showing side chains of residues within 5 Å of bound Taxol (green sticks), viewed parallel to the membrane plane. EM density is shown as a blue mesh, contoured at 6σ. (D) Ribbon representation of TM4 (yellow) and TM10 (orange) adopting kinked conformation, with Taxol located in the center of the occluded cavity. EM density after nanodisc subtraction is contoured at 8σ. (E) Interactions between Taxol and ABCB1 side chains. Nonbonded interactions are represented by spoked arcs and hydrogen bonds are indicated by dashed green lines. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; F, Phe; I, Ile; L, Leu; M, Met; Q, Gln; S, Ser; W, Trp; and Y, Tyr.



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We observed two main conformations in our single-particle cryo-EM analysis (fig. S1). The highest-resolution structure (Fig. 1B) revealed an occluded conformation with density covering a single Taxol molecule (Fig. 1C) in a central cavity formed by the closing of a gate region consisting of TM4 and TM10 (Fig. 1D). The NBDs were closer together than in previously determined, inward-open apo structures of mouse ABCB1 (11, 14–16) and more closely resembled those of disulfide-trapped human-mouse chimeric ABCB1 (ABCB1_{HM}) structures (11) despite the absence of nucleotides or disulfide cross-linking. The second conformation revealed a slightly larger separation of the NBDs and poorly ordered TM4 and TM10 segments. In this conformation, the cytoplasmic gate to the drug-binding cavity is open. Our results demonstrate that binding of Taxol to ABCB1 induces an occluded conformation and a concomitant closure of the inter-NBD gap, which is in line with earlier mutagenesis and biochemical work (17, 18). The central pocket of Taxol-bound ABCB1 is lined by amino acid residues from all 12 TM helices. Whereas the density for interacting residues was well defined, that of the Taxol molecule was less clear, suggesting the possibility of multiple binding modes. The orientation of Taxol shown in Fig. 1, C and E, had the strongest density assigned to the tetracyclic core (baccatin III) with the cyclooctane ring in a crown conformation. The peripheral moieties displayed conformational heterogeneity, and their placement was guided by fitting the Y-shaped tail of the molecule to avoid steric clashes with neighboring side chains. Given its volume, only one Taxol molecule can bind to the central cavity of ABCB1, and occlusion of the drug-binding pocket is triggered irrespective of which binding mode the molecule adopts. The drug-binding cavity of ABCB1 is globular in shape, in contrast to the flatter, slit-like drug-binding pocket previously visualized in the human multidrug transporter ABCG2 (19, 20). This is in line with the finding that Taxol cannot bind to ABCG2 or modulate its activity (21, 22). A comparison of the substrate- and inhibitor-bound structures of these two key human multidrug exporters therefore allows us to rationalize their divergent substrate specificities.

For the zosuquidar-bound structure, we used a hydrolysis-deficient variant of ABCB1_{HM} harboring an E→Q mutation in the walker-B motif (ABCB1_{HM-EQ}) and added ATP to ensure that zosuquidar had indeed trapped ABCB1 in an inhibited state, given that, in the presence of ATP and Mg²⁺, this ABCB1 variant had been shown to adopt a closed NBD conformation coupled to a closed TMD conformation with a collapsed translocation pathway (23). Our zosuquidar-inhibited ABCB1_{HM-EQ} structure (Fig. 2A and figs. S3 to S5) displayed a conformation similar to that of Taxol-bound ABCB1—with the TMDs forming an analogous occluded cavity—but containing two bound zosuquidar molecules. We again observed two main ABCB1 conformations, characterized by a distinct degree of NBD opening. However, unlike for Taxol, both conformations revealed bound

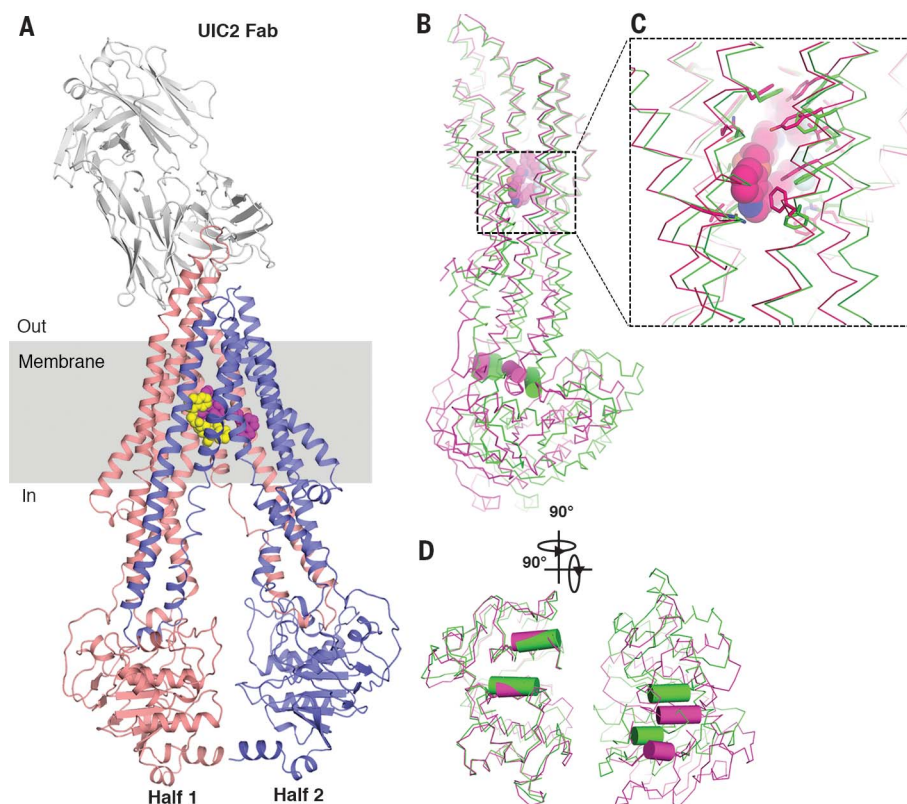


Fig. 2. Comparison of zosuquidar- and Taxol-bound ABCB1. (A) Cartoon of zosuquidar-bound ABCB1_{HM-EQ} structure with zosuquidar molecules shown as yellow and magenta spheres. The N- and C-terminal halves of ABCB1 are colored pink and blue, respectively. (B to D) Superposition of Taxol-bound human ABCB1 (green ribbon) and zosuquidar-bound ABCB1_{HM-EQ} (magenta ribbon) with intracellular helices interacting with NBDs shown as cylinders. Zosuquidar molecules are shown as spheres.

zosuquidar molecules. The density for zosuquidar was better defined than that for Taxol and allowed unambiguous placement in a specific binding mode (figs. S4 and S5, B and C). This likely stems from the increased contact area (buried surface) between the two zosuquidar molecules and ABCB1 (~1000 Å²) compared with Taxol (~800 Å²) as well as the intermolecular contact between the two zosuquidar molecules themselves (interface surface area of ~190 Å²). The orientation and binding interactions of the zosuquidar molecules are similar to those previously observed in the disulfide-trapped, detergent-solubilized ABCB1_{HM} structure, suggesting that the opposite effect of zosuquidar on the ATPase rate of ABCB1 in lipid bilayers (reduction of ATPase activity) or detergent solution (stimulation) is not attributable to distinct binding sites of zosuquidar in these lipidic environments.

Zosuquidar and Taxol binding to the same pocket raises the key questions of how ABCB1 distinguishes transport substrates from inhibitors and how these compounds exert opposite effects on ATPase activity. To explain the polyspecificity of ABCB1, plasticity of the drug-binding pocket in terms of side-chain and backbone rearrangements to accommodate distinct substrates has often been invoked (24, 25). We therefore superimposed Taxol-bound and zosuquidar-bound

ABCB1 and found that the main-chain and side-chain conformations of the residues surrounding bound drugs are largely similar (Fig. 2, B to D, and fig. S6). However, there were a number of small but notable structural differences localized primarily in the second half of the transporter. As seen in Fig. 2B, the subtle changes originate at the drug-binding site and are transmitted and amplified via the helix pair TM7-TM8 and TM12 to NBD2 (Fig. 2, C and D). They lead to an outward shift of intracellular helix 2 (coupling helix 1) in zosuquidar-bound ABCB1, increasing the distance between the NBDs and offering a plausible explanation for the reduced ATPase activity in the presence of zosuquidar. Thus, our results demonstrate that plasticity occurring within the confines of the occluded drug-binding pocket can be linked to NBD movement and ATPase activity of ABCB1.

The function of ABCB1 is known to be modulated by the lipidic membrane. As was observed in the structures of nanodisc-reconstituted ABCG2 (19), we found ordered cholesterol and phospholipid molecules bound to the transmembrane region of ABCB1 (Fig. 3 and fig. S2). At the level of the outer membrane leaflet, a ring of ordered cholesterol molecules is bound to surface grooves on ABCB1, and specific interactions include hydrogen bonds with the hydroxyl group of cholesterol

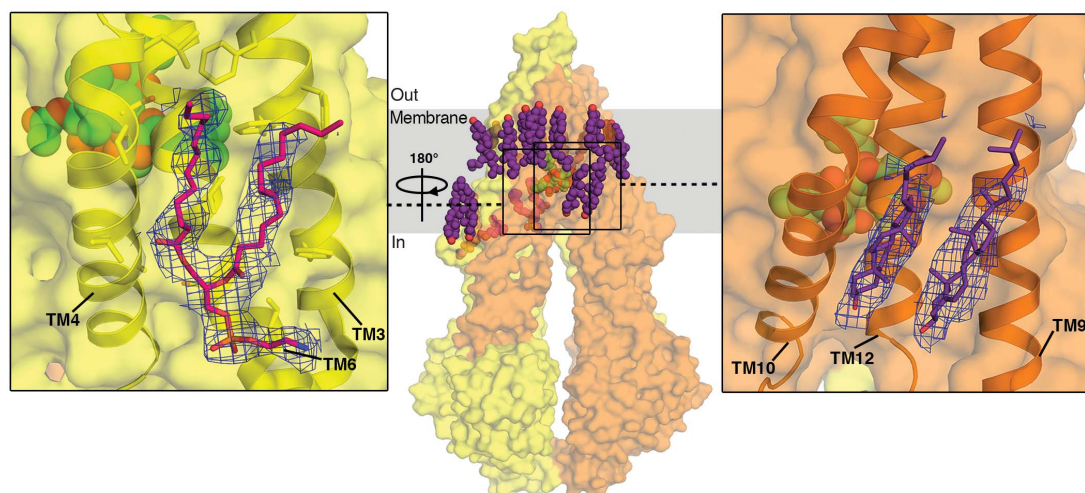
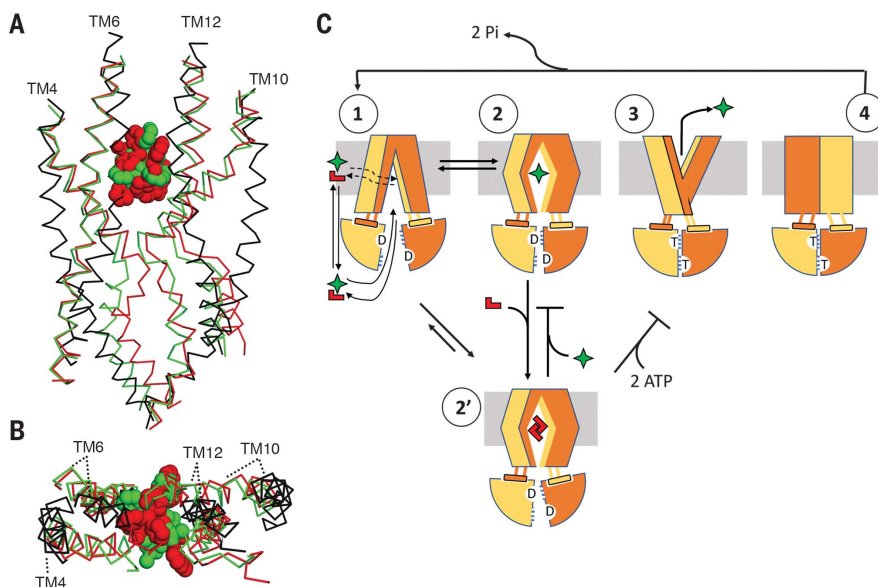


Fig. 3. Phospholipids and cholesterol bound to ABCB1. (Center) Surface representation of Taxol-bound human ABCB1 showing bound lipid (phosphatidylethanolamine, magenta spheres) and cholesterol molecules (purple spheres). Zoom-in panels show details of binding sites of (left) phospholipid (magenta sticks) and (right) cholesterol (purple sticks) at the level of the inner leaflet, near the kinking TM helices TM4 and TM10. EM density (blue mesh) is contoured at 6σ .

Fig. 4. Proposed mechanism of P-glycoprotein/ABCB1 substrate transport and small-molecule inhibition. (A) Side view of select TM helices of ABCB1 after superposition of the drug-bound, occluded conformation (green and red ribbons for Taxol- and zosuquidar-bound structures, respectively) with the previously reported ATP-bound posttranslocation state (black ribbons). Taxol and zosuquidar molecules are shown as green and red spheres, respectively. (B) Same as (A) but viewed from the cytoplasm. (C) Schematic of proposed ABCB1 transport cycle in the presence of substrate (Taxol, green star) and inhibitor (zosuquidar, red L-shape). ATP and adenosine diphosphate are indicated by T and D, respectively, whereas the dashed lines in the NBDs represent ATP-binding elements required for NBD dimerization and ATP hydrolysis. Major conformational states are represented by circled numbers. State 1: apo state [Protein Data Bank (PDB) IDs 4M1M and 4QNH, among others]. States 2 and 2': drug- and inhibitor-bound, this study. State 3: proposed outward-facing conformation based on Sav1866 structure (30). State 4: collapsed posttranslocation state (PDB ID 6C0V). Pi, inorganic phosphate.



and stacking interactions with aromatic side chains, as observed for other cholesterol-protein interactions (26). At the level of the inner leaflet, density compatible with a bound phospholipid and cholesterol was observed in a membrane-exposed pocket near TM3, TM4, and TM6 (Fig. 3, left panel) and at the pseudosymmetrically related site near TM9, TM10, and TM12 (Fig. 3, right panel). Because the phospholipid- and cholesterol-binding sites are formed by the kinking of TM4 and TM10 in response to drug and inhibitor binding, our observation suggests a direct mechanism of ABCB1 modulation by inner leaflet lipids.

When combined with the previously reported structure of ATP-bound ABCB1_{EQ} (23), our results offer a structural mechanism both for drug extrusion in a transport cycle and competitive inhibition of this reaction by small-molecule inhibitors. Formation of the closed ATP-bound NBD dimer triggers conformational changes in TM4 and TM6 from TMD1 as well as the symmetrically related TM10 and TM12 from TMD2, generating a steric clash with bound drugs (Fig. 4, A and B). This suggests that a peristaltic mechanism contributes to the extrusion of bound substrate during the transport cycle (Fig. 4C). Competitive inhibitors such as zosuquidar likely

function by arresting the transporter in an occluded conformation, thus (i) restricting access to the substrate binding site, (ii) preventing NBD closure and consequently inhibiting ATPase activity, and (iii) preventing a transition to an outward open state. Our data are in line with proposed mechanisms of relaying long-range structural changes upon substrate and inhibitor binding to the NBDs (17), as well as subtle induced-fit type rearrangements of a dynamic binding pocket (18, 24). In contrast to transport substrates, where multiple holo and apo forms of ABCB1 likely coexist, inhibitors have fewer or a single binding mode, fill the drug-binding cavity

more completely, and form a larger number of contacts with ABCB1. This suggests that, in a continuum of substrates and inhibitors, three-dimensional shape complementarity and the strength of the contacts rather than distinct binding sites are key determinants distinguishing substrates from inhibitors. Although the functional modulation of ABCB1 by cholesterol and lipids has previously been demonstrated (12, 27–29), our results illustrate specific interactions and binding sites in cholesterol-containing lipid bilayers.

Finally, given that the structures of Taxol- and zosuquidar-bound ABCB1 reveal critical interactions in a key state of the transporter, our results may allow medicinal chemists and computational biologists to exploit structural insight into ABCB1 to guide the design of drugs or inhibitors.

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SUPPLEMENTARY MATERIALS

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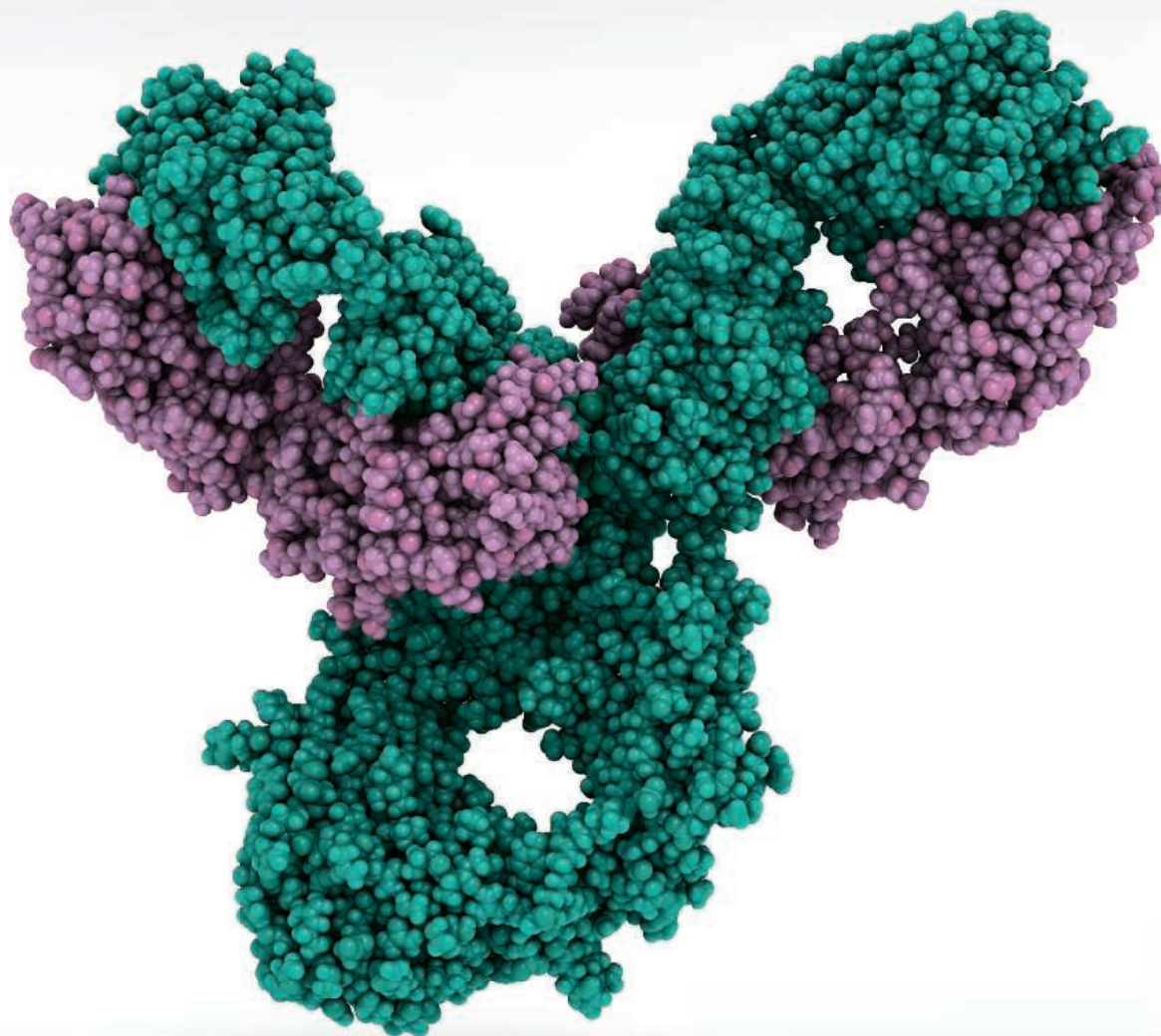
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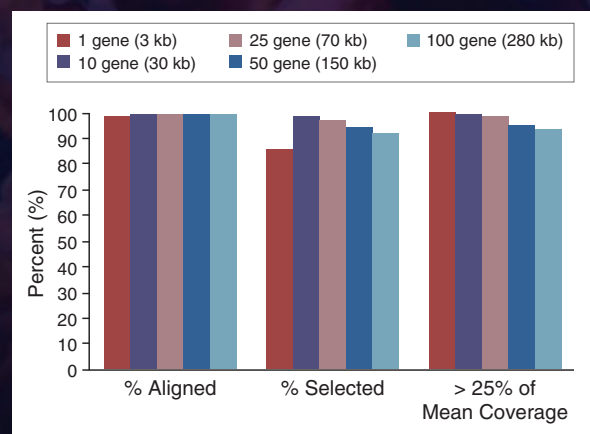
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ELECTROPHYSIOLOGIST/ BIOPHYSICIST: ION CHANNELS AND HYPEREXCITABILITY DISORDERS

The Center for Neuroscience & Regeneration Research at Yale University has an opening for an ion channel physiologist, to join multidisciplinary research group studying properties and roles of ion channels associated with hyperexcitability disorders such as pain. Doctoral degree and substantive experience at channel physiology are essential. Experience in slice recording, channel pharmacology and/or optical imaging would be a plus. Superb opportunity to work collaboratively within a multidisciplinary group with molecular and cell biologists, pharmacologists, pain researchers, biophysicists and physiologists using voltage-clamp, current-clamp, dynamic clamp and optical methods to study ion channels in health and disease. See Dib-Hajj and Waxman *Annu Rev Neurosci* 2019. DOI: 10.1146/annurev-neuro-070918-050144; Yang et al, *Trends Pharmacol Sci* 2018; 39: 258-275. DOI: 10.1016/j.tips.2017.11.010.

Send C.V., three letters of reference, and statement of interests to Stephen.waxman@yale.edu.

*Women and members of underrepresented minority groups are encouraged to apply.
Affirmative Action/Equal Opportunity Employer.*

Postdoctoral Traineeship: Maternal and Child Nutrition

The Division of Nutritional Sciences at Cornell University seeks to fill an NIH-funded postdoctoral fellowship in Maternal and Child Nutrition. The successful applicant will have a Doctoral-level degree and a record of accomplishment in a relevant field and be committed to a career of research in Maternal and Child Nutrition. Research will be supervised by one or more of 13 faculty mentors. Opportunities are available for classroom-based training and professional development. U.S. citizenship or permanent residency is required. This position is available beginning May 1, 2019.

Qualified individuals must apply through Academic Jobs Online (AJO) <https://academicjobsonline.org/ajo/jobs/13159> and must submit: (a) cover letter, (b) curriculum vitae, (c) statement of research interests, and (d) 3 reference letters (to be submitted through AJO by the reference writers). Questions in advance of application should be sent to Professor Kathleen Rasmussen at Kathleen.Rasmussen@cornell.edu. Closing date: March 15, 2019.



Diversity and Inclusion are a part of Cornell University's heritage. We're an employer and education recognized for valuing AA/EEO, Protected Veterans, and Individuals with Disabilities.

Director, Public Health Research Institute

Rutgers New Jersey Medical School (NJMS) invites applicants for the position of Director of the Public Health Research Institute (PHRI) center. We are seeking an individual with outstanding leadership qualities and a record of accomplishment as a well-funded biomedical researcher to direct a major biomedical research center at NJMS.

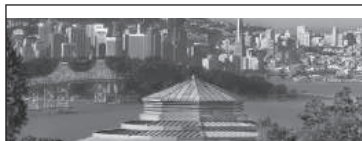
Over 120 scientists at PHRI pursue infectious disease research, focusing on HIV and other viruses, mycobacteria and other bacterial pathogens, and opportunistic fungi.

The successful candidate should be a leader committed to maintaining PHRI as a global leader in basic and translational science, supported by federally funded research and strategic initiatives in private partnerships.

The center is located on the Rutgers New Jersey Medical School Newark Campus in the International Center for Public Health (ICPH) building, which is home to the NJMS Department of Microbiology, Biochemistry and Molecular Genetics, a National Regional Biocontainment Laboratory, BSL2 laboratories and BSL3 small-animal vivarium space, and the Global Tuberculous Center. PHRI is one of three major centers within the Rutgers Biomedical Health Sciences Institute for Infectious and Inflammatory Diseases. Further information about PHRI can be found at: <https://phri.njms.rutgers.edu/>

Interested individuals must apply on-line at <http://jobs.rutgers.edu/> posting and submit a Curriculum Vitae and letter of inquiry to: **Vivian Bellofatto, Ph.D., Professor and Chair (Interim), Department of Microbiology, Biochemistry & Molecular Genetics; Chair, Search Committee for Director, Public Health Research Institute, c/o Michael Petti, Executive Assistant to the Dean, Rutgers New Jersey Medical School, 185 south Orange Avenue, MSB C-671, Newark, NJ 07101-1709; E-mail: petime@njms.rutgers.edu**

An equal opportunity and affirmative action employer, Rutgers, The State University of New Jersey, is committed to building a diverse community and encourages women, minorities, veterans and individuals with disabilities to apply. For additional information, please see our Non-Discrimination Statement.

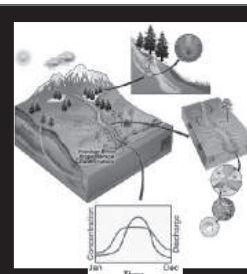


Hydrologist

The Earth and Environmental Sciences (EESA.lbl.gov) of Berkeley Lab is advancing and integrating diverse expertise to accelerate scientific discoveries and their translation into scalable solutions that simultaneously sustain the Earth's environment and the growing need for energy and water resources.

We seek an outstanding Earth Scientist with a record of innovative research in hydrology. Aligned with EESA's 'Future Water' and possibly 'Sustainable Earth' strategic directions, we particularly encourage applicants who develop and utilize novel field methods and experiments with theory to advance understanding of multi-scale, multi-phase behavior of hydrological systems. Topics of interest include, but are not limited to: watershed response to extreme events and climate change; critical zone ecohydrology and hydrogeochemistry; aquifer storage and recovery; and bedrock flow and reactions relevant to subsurface energy strategies.

The position is open at the Research and Staff Scientist levels. The incumbent is expected to take advantage of world-class experimental facilities at Berkeley Lab and develop active collaboration with UC Berkeley. The incumbent may also be considered for leadership roles within EESA.



Berkeley Lab, located next to UC Berkeley, is part of the stimulating San Francisco Bay Area innovation ecosystem. The region is also recognized for offering a high quality of life, having both abundant natural beauty and exciting urban surrounds.

To apply online at <http://jobs.lbl.gov>, please select "Search Jobs", enter 86204 in the keyword search field, and follow the online instructions to complete the application process.

LBLN is an Equal Opportunity/Affirmative Action Employer.



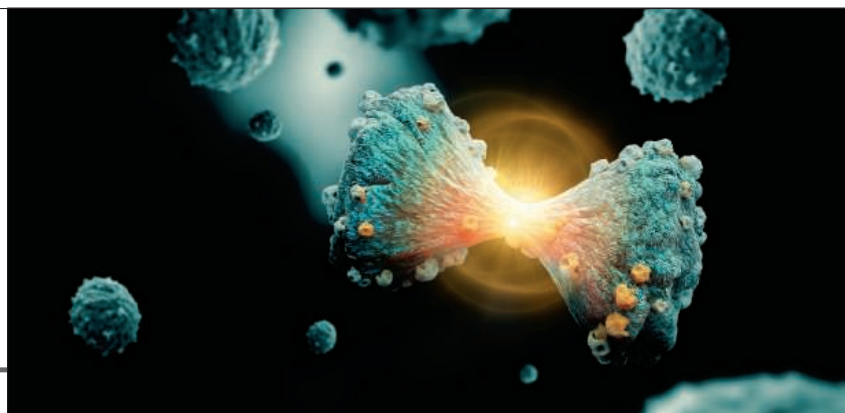
CAREER FEATURE:

Cancer Research

Issue date: March 15

Reserve space by February 28

Ads accepted until Mar 8 if space allows



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UTHealth

The University of Texas
Health Science Center at Tyler

The Texas Lung Injury Institute at the University of Texas Health Science Center at Tyler invites applications from outstanding scientists for state funded faculty positions at all levels to conduct independent research. The research of the applicant should be in the areas of pulmonary fibrosis, aspects

of acute lung injury, airways disease, pulmonary infectious diseases or trauma. Applicants should have extramural funding and at the junior level, K99/00 applicants are particularly encouraged and will receive strong mentorship.

The mission of the research program at UT Health Science Center at Tyler is to create an outstanding research community that links basic and clinical science. The institution has established research programs in lung injury/repair, cell motility and cytoskeletal rearrangement and pulmonary infectious diseases. The successful candidate is encouraged to interact with investigators in these areas. A strong track record of achievement is required. Teaching in the biotechnology graduate program is encouraged. Endowment, university and philanthropic funds are available to support successful candidates.

Our institution is growing rapidly and is now a part of a billion-dollar ten hospital system with a strong public health program. Substantive resources have been allocated to build its basic and translational research portfolio, including commercialization. The institution has recently set up a state-of-the-art cellular and molecular imaging facility, has purchased a new small animal spiral PET/CT scanner, is in the process of renovating all of laboratories in the Biomedical Research Building and has expanded its vivarium resources. The Tyler area is beautiful, offers strong educational options, cultural events and proximity to Dallas.

Applicants should submit their CV, a statement of accomplishments, future research plans and the names of three references to: **Dr. Steven Idell, Senior Vice President for Research and Dean, School of Medical Biological Sciences, The University of Texas Health Science Center at Tyler, 11937 US Highway 271, Tyler TX 75708-3154; E-mail: Brigitte.Tolson@uthct.edu.**

As an Equal Opportunity Employer, the University will, in accordance with State and Federal law and regulations, provide equal opportunity in all employment related activities without regard to race, color, religion, national origin, gender, age, disability, sexual preference, or status as a disabled veteran or a veteran of the Vietnam Era. The University requires pre-employment drug screening and criminal background check on all hires. Specific job requirements or physical location of some positions allocated to this classification, may render this position security sensitive, and thereby subject to the provisions of Section 51.215, Texas Education Code.

PRIZES



The 2019 (35th) International Prize for Biology

Calling for Nominations

This year's research field: **Biology of Insects**

Please access at: <http://www.jsps.go.jp/english/e-biol>

Deadline: April 19, 2019

- The International Prize for Biology was established in 1985 to commemorate the 60-year reign of Emperor Showa and his longtime devotion to biological research.
- The Prize is awarded each year to an individual who has made an outstanding contribution to the advancement of basic research in a field of biology.
- The Prize shall consist of a medal and a prize of 10 million yen.

Recent Years Prize Winners



2018
Dr. Andrew H. Knoll
(Paleontology)



2017
Dr. Rita R. Colwell
(Marine Biology)



2016
Dr. Stephen P. Hubbell
(Biology of Biodiversity)



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This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 801199.



EUROPEAN UNION

By Jessica Sagers

Waiting for reimbursement

It has been an exciting and productive conference. As a fifth-year Ph.D. student, I am less nervous when I present my work than I used to be, and I finally feel I fit in as a scholar. But when my abstract was accepted in the fall, I had to consider something completely unrelated to my science: whether I could afford to go. Carefully, I did the math. Registration, fees, air travel, lodging, and food would exceed \$1000. My fellowship comes with \$1200 that I can use for conference travel, among other project-related expenses. On top of that, the conference granted my presentation a \$500 travel award. I should be able to cover the costs—eventually.

I can't access these funds until after the conference, long after paying for registration and flights. So, for the past 3 months, I have been out a significant chunk of cash. This is money that I can't really spare, as most of my modest graduate stipend goes to day-to-day necessities—including food, housing, and utilities. I'll submit my reimbursement forms as soon as possible once I get home. But I know from experience that sometimes payments are extremely delayed, even after the forms are filed. All I can do is hope the process will go smoothly this time—and know that I'll have to advocate for myself and micromanage every step if it doesn't.

Still, I count myself luckier than some. I can draw on savings I was able to build up while I was in college. I attended a budget-conscious institution in my home state, cobbled together scholarship funds to cover my tuition and fees without help from my parents, lived in cheap apartments, and worked multiple jobs so that I could save. That means that these days, though sometimes I have to get creative, I can generally make conference expenses work. But I incur these expenses in a sometimes-scary bet against the bank account I use to pay my rent and keep my apartment warm.

Requiring students to ante up conference funds up front without the hope of being reimbursed for months makes academia less welcoming for scientists who are financially disadvantaged. Yet universities and funding agencies seem unwilling or unable to do much to change the system. Perhaps changing the status quo requires too much work. Maybe it's not a priority because many students come from privileged backgrounds that insulate them from the issue. Students for whom this is a real problem may feel ashamed to ask for help and be treated as an exception.



"I incur these expenses in a sometimes-scary bet against [my] bank account."

I've tried to work with administrators to find reasonable solutions, but the results have been mixed at best. Last summer, for example, I had the opportunity to attend a prestigious weeklong conference, supported by a competitive grant. But, as always, all funds were due up front at registration. Assuming the financial and psychological burden of these expenses—thousands of dollars—felt like more than I could take on, especially after already putting in substantial time and intellectual effort to secure the grant.

After negotiating with administrators, I thought I came up with a solution. The granting institution set up an account with the hospital that housed my lab so that I could pay my expenses directly with the funds I had been awarded. But when I tried to register for the conference, I

hit an unexpected snag: All payments needed to be made by credit card. I nearly cried. Embarrassingly, the balance due exceeded my card's limit. After some desperate emails, an administrator agreed to extend the credit line on an existing department card so that I could use it. But she made sure I understood that this was not something they normally did and that I shouldn't make a habit of these types of requests.

As for my most recent conference expenses, I was relieved to find out a few weeks ago that I'd receive a check for my \$500 travel grant on site at the conference. I probably won't receive my reimbursement for the rest of the conference expenses until April (assuming all goes well). Until then, I'm gearing up to devote a significant amount of mental energy to keeping a careful eye on my bank account and credit cards. ■

Jessica Sagers is a Ph.D. candidate at Harvard University. Send your career story to SciCareerEditor@aaas.org.